Unravelling the mystery of the shark genus *Mustelus* in southern Africa using a multidisciplinary approach

by

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Dissertation presented for the degree of
Doctor of Philosophy (Science)

at
Stellenbosch University
Genetics Department, Faculty of Science

The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF

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December 2017

Declaration

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Date: December 2017

Abstract

Multidisciplinary approaches have previously offered some alternative innovative ways of addressing classical ecological questions while providing novel insights into behaviour and biology of elasmobranch species. The species-rich shark genus *Mustelus*, or smoothhounds (smoothhound sharks), is one of the most bio-economically important groups of elasmobranchs in the world's oceans. Despite the commercial value of *Mustelus*, its systematics remains largely unresolved and the knowledge on the copulating and dispersal strategies of species of *Mustelus* is scarce. Here, a multidisciplinary approach – molecular, morphology and histology – with different methods of analysis on various spatial scales was used.

First, this study investigated the evolutionary origin of the shark genus *Mustelus* in southern Africa using molecular phylogenetic and statistical biogeography approaches. Results gave strong support for a northern hemisphere origin of southern African *Mustelus* species, and that the radiation of *Mustelus* in this region was primarily driven by long-distance dispersal. The monophyly of expanded *Mustelus* indicated that southern African species of the genus arose from at least two separate colonisation events from the Northern Hemisphere.

On a microevolutionary scale, a comparative population genetics approach was used to gain insight into spatial genetic structure and dispersal patterns in species of *Mustelus* (*M. mustelus* and *M. palumbes*) and other co-distributed demersal sharks (*Galeorhinus galeus* and *Triakis megalopterus*) characterised by assorted life histories, habitat preferences, and dispersal behaviour. Based on novel Next Generation Sequencing (NGS)-mined microsatellites, the null hypothesis of genetic homogeneity was rejected for all species investigated except for *T. megalopterus*. Most noteworthy is that the coalescent analysis of migration supported asymmetric gene flow from the Indian to the Atlantic Ocean, concordant with the Atlantic Ocean–Indian Ocean

connection via Agulhas leakage proposed for many marine species along the South African coast.

In terms of fisheries forensics, a dermal denticle identification key guide and two molecular assays (a microsatellite panel and High-resolution melting assay) were successfully developed for species identification of southern African *Mustelus* (*M. mosis*, *M. mustelus* and *M. palumbes*) and three other shark species (*Galeorhinus galeus*, *Scylliogaleus quecketti* and *Triakis megalopterus*) commonly confused with species of *Mustelus* in the region. Additionally, a SNP discovery and genotyping pipeline was optimised that could in future be used to obtain genome-wide data that will enable population genetic and demographic processes of the study species to be assessed more accurately. Lastly, evidence of sperm storage in female common smoothhound sharks was reported for the first time using a histological approach. The molecular analysis of a single common smoothhound litter also hinted at the within-species variation in the presence and frequency of multiple paternity previously reported for elasmobranchs.

Overall, this study provides the most comprehensive set of conservation genetic resources for the common smoothhound shark to date. The results provide novel insights into the conservation biogeography, species identification and ecology of dispersal as well as mating behaviours in species of *Mustelus*. This will help inform existing and ongoing management and conservation efforts for smoothhound sharks occurring in southern Africa.

Opsomming

Multi-dissiplinêre benaderings bied alternatiewe innoverende maniere om klassieke ekologiese vrae aan te spreek, terwyl dit nuwe insig in gedrag en biologie van kraakbeenagtige visspesies verskaf. Die spesie-ryk haai genus *Mustelus*, of gladhonde (gladhondhaaie), is een van die mees bio-ekonomiese belangrike groepe van die kraakbeenagtige visspesies in die wêreld se oseane. Ten spyte van die kommersiële waarde van *Mustelus*, bly die genus se klassifikasie grootliks onopgelos en die kennis oor die kopulering en verspreidingstrategieë van spesies van *Mustelus* is skaars. Hier word 'n multi-dissiplinêre benadering – molekulêre, morfologie en histologie – met verskillende metodes van analiese op verskeie geografiese skale gebruik.

Eerstens het hierdie studie die evolusionêre oorsprong van die haai genus *Mustelus* in suidelike Afrika ondersoek deur gebruik te maak van molekulêre filogenetiese en statistiese biogeografiese benaderings. Resultate het sterk steun verleen vir oorsprong vanaf die noordelike halfrond vir die Suider-Afrikaanse *Mustelus* spesies, en dat die radiasie van *Mustelus* in hierdie streek hoofsaaklik deur langafstandverspreiding gedryf is. Die monofilie van uigebreide *Mustelus* het aangedui dat die Suider-Afrikaanse spesies van die genus ontstaan het uit ten minste twee afsonderlike kolonisasie gebeure uit die noordelike halfrond.

Op 'n mikroevolusionêre skaal, was 'n vergelykende populasiegenetika-benadering gebruik om insig te verkry in geografiese genetiese struktuur en verspreidingspatrone in *Mustelus* (*M. mustelus* and *M. palumbes*) en ander demersale haai spesies (*Galeorhinus galeus* and *Triakis megalopterus*) gekenmerk deur verskillende lewenssiklusse, habitatvoorkeure en verspreidingspatrone. Gebaseer op unieke Volgende Generasie Volgorderbepalings (VGV)-gemynde mikrosatelliete, is die nulhipotese van genetiese homogeniteit verwerp vir alle spesies ondersoek hier behalwe vir *T. megalopterus*. Mees opmerklik is dat die analise van migrasie assimetriese geenvloei van die Indiese na die Atlantiese Oseaan ondersteun,

ooreenstemmend met die Atlantiese Oseaan-Indiese Oseaan verbinding via Agulhas lekkasie voorgestel vir baie mariene spesies langs die Suid-Afrikaanse kus.

In terme van vissery forensiese navorsing, was ‘n suksesvolle morfologiese identifikasie gids en twee molekulêre toetse (‘n mikrosatelliet paneel en hoë resolusie smelting toets) ontwikkel vir spesies indentifikasie van Suider-Afrikaanse *Mustelus* (*M. mosis*, *M. mustelus* and *M. palumbes*) en drie ander haai spesies (*Galeorhinus galeus*, *Scylliogaleus quecketti* and *Triakis megalopterus*) wat algemeen verwar word met *Mustelus* spesies in die streek. Daarbenewens is ‘n SNP merker ontwikkeling pyplyn geoptimiseer wat in die toekoms gebruik kan word om genoomwye data te verkry wat assessering van populasiegenetiese en demografiese prosesse van die studie spesie sal vergemaklik. Laastens, bewyse van spermberging in vroulike *M. mustelus* is vir die eerste keer aangemeld met behulp van 'n histologiese benadering. Die molekulêre analise van 'n enkele *M. mustelus* broeisel het ook aangedui op die binne-spesie variasie in die teenwoordigheid en frekwensie van veelvuldige vaderskap wat voorheen vir kraakbeenagtige visspesies gerapporteer is.

In die geheel bied hierdie studie tot op hede die mees omvattende samestelling van bewaringsgenetiese hulpbronne vir die *M. mustelus* haai. Die resultate bied nuwe insigte in die bewaringsbiogeografie, spesie identifikasie en ekologie van verspreiding asook paring gedrag in *Mustelus* spesies. Dit sal help om bestaande en deurlopende bestuurs- en bewaringspogings vir hondhaaie wat in Suider-Afrika voorkom in te lig.

Acknowledgements

I wish to express my sincere gratitude and appreciation to the following persons and institutions (in no particular order):

- Dr Aletta Bester-van der Merwe – my maker – for being an outstanding supervisor. When it comes to me, she wears many hats; teacher, mentor, guardian, psychologist and the list goes on.

- Colleagues (and friends) who afforded me immense support and courage when life threw me its curve balls: Margarete Sittmann, Katie Gledhill, Melt Hugo and Gibbs Kuguru.

- Family who had to endure my absence during the tenure of my doctoral candidature. I love you and appreciate everything you have done for me.

- Charlene Da Silva and the rest of the team at the Department of Agriculture, Forestry and Fisheries (DAFF), South Africa, for providing shark specimens, tissue samples and facilities to perform shark dissections. Importantly, Charlene Da Silva is acknowledged for her instrumental role during the preparation of several manuscripts emanating from this dissertation and other collaborations.

- Local collaborators and field scientists who assisted with the collection of southern African samples vital to the completion of my dissertation: Dr Michelle Soekoe (Rhodes University), Drs Sabine Wintner and Matt Dicken (KZN Sharks Board), Dr Enrico Gennari (Oceans Research), Gibbs Kuguru (White Shark Africa), South African Shark Conservancy (SASC) team, Grant van der Heever (University of the Western Cape) and Bruce Mann (Oceanographic Research Institute).

- International collaborators for providing samples which made Chapter 3 possible: Dr Edward D. Farrell (University College Dublin, Ireland), Dr Jessica J. Boomer (Macquarie University, Australia), Prof. Stefano Mariani (University of Salford, Manchester), Dr Ilaria M. Marino and Prof. Lorenzo Zane (University of Padova, Italy), Dr Ana Verríssimo (Research Center for Biodiversity and Genetic Resources, Portugal), Dr Chrysa Gubili (University of Salford, Manchester) and Dr Mikhail V. Chesalin (Fisheries Research Center Salalah, Oman).

- Anelda van der Walt for training me in Next-Generation Sequencing (NGS) data analysis, affording me the bioinformatics skills set which made my Chapters 4 and 5 possible.

- Dirk Aldrich (Stellenbosch University, South Africa) for sharing his scientific wisdom which assisting me in successfully carrying out the high-resolution melting analysis reported in Chapter 6.

- Prof. Johannes H. van Wyk (Stellenbosch University, South Africa) for providing the expertise, resources and infrastructure for histology work conducted in Chapter 7 and for assisting with the preparation of the manuscript reporting on the findings of the chapter.

- Profs Per Palsbøll (University of Groningen, Netherlands) and Rouvay Roodt-Wilding (Stellenbosch University, South Africa) for providing valuable and constructive comments on an earlier version of the review manuscript presented in Chapter 2. Also to Gavin Naylor (Florida State University, United States) for valuable discussions on the current phylogenetic systematics of the genus *Mustelus*, and Dr Melissa Giresi (Texas A&M University, United States) for sharing her findings on the population genetics of *Mustelus canis*.

- Funding which saw through this dissertation was provided by the Save Our Seas Foundation, National Research Foundation of South Africa and the Genetics Department, Stellenbosch University.

Preface

This dissertation is presented as a compilation of eight chapters. Below are my scientific contributions during the tenure of my doctoral candidature (2015-2017):

1. **Published or submitted papers, to date, directly emanating from the work presented in this dissertation:**

Maduna SN, Rossouw C, Da Silva C, Soekoe M, Bester-van der Merwe AE. 2017. Species identification and comparative population genetics of four coastal houndsharks based on novel NGS mined microsatellites. 2017. *Ecology and Evolution* 7: 1462–1486. [**Chapter 4 and 6**]

Maduna SN, Bester-van der Merwe AE. In press. Molecular research on the systematically challenging smoothhound genus *Mustelus*: a synthesis of the past 30 years. *African Journal of Marine Science* doi: 10.2989/1814232X.2017.1394365. [**Chapter 2**]

Maduna SN, van Wyk JH, Da Silva C, Gennari, Bester-van der Merwe AE. (second round revision). Evidence for sperm storage in common smoothhound shark (Mustelus mustelus) and paternity assessment in a single litter from South Africa. *Journal of Fish Biology*. Initial submission on 15 March 2017. [**Chapter 7**]

2. **Papers in preparation to be submitted to respective journals directly emanating from the work presented in this dissertation:**

Maduna SN, Farrell ED, Boomer JJ, Da Silva C, Verríssimo A, Gubili C, Soekoe M, Marino IAM, Zane L, Wintner SP, Mariani S, Bester-van der Merwe AE. North of the wall: the historical biogeography of the shark genus *Mustelus* with particular reference to species from southern Africa. Target journal: *Diversity and Distributions* [**Chapter 3**]

Maduna SN, Hull KL, Da Silva C, Soekoe M and Bester-van der Merwe AE. When morphometry meets genetics: Development of species identification methods of houndsharks (Carcharhiniformes: Triakidae) from southern Africa. Target journal: *Fisheries Research* [**Chapter 6**]

3. Conference presentations:

Maduna SN*, Bester-van der Merwe AE. Oral presentation: Unraveling the mystery of the shark genus *Mustelus* in southern Africa using a multidisciplinary approach: implications for management and conservation. *4th Southern African Shark and Ray Symposium*. September 2017. Hermanus, South Africa.

Hull KL*, **Maduna SN**, Da Silva C, Dicken ML, Marino IAM, Zane L, Bester-van der Merwe AE. Oral presentation: Global population structure and mitogenome assembly of the common smoothhound shark, *Mustelus mustelus*. *4th Southern African Shark and Ray Symposium*. September 2017. Hermanus, South Africa.

Maduna SN, Hull KL*, Da Silva C, Soekoe M, Bester-van der Merwe AE. Poster presentation: Development of molecular identification methods of houndsharks (Carcharhiniformes: Triakidae) from southern Africa. *3rd Joint SASBi-SAGS Congress*. September 2016. Durban, South Africa: PG2-105 *(Awarded best poster presentation)*

Maduna SN*, Rossouw C, Da Silva C, Soekoe M, Bester-van der Merwe AE. Oral presentation: Discovery and application of molecular markers in a bio-economically important demersal shark *Mustelus mustelus* using next-generation sequencing technology. *3rd Joint SASBi-SAGS Congress*. September 2016. Durban, South Africa: 67

Maduna SN*, Farrell ED, Boomer JJ, Da Silva C, Soekoe M, Wintner SP Mariani S, Bester-van der Merwe AE. Oral presentation: The historical biogeography of the systematically challenging smoothhound shark genus *Mustelus* in southern Africa. *3rd Joint SASBi-SAGS Congress*. September 2016. Durban, South Africa: 49

Maduna SN*, Farrell ED, Boomer JJ, Da Silva C, Soekoe M, Wintner SP Mariani S, Bester-van der Merwe AE. Oral presentation: From whence they came: Unravelling the evolutionary origin of the shark genus *Mustelus* in southern Africa using a molecular phylogenetic approach. *3rd Southern African Shark and Ray Symposium*. September 2015 False Bay, Cape Town, South Africa.

[**Presenting author*]

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%	Percentage
(Pty)	Ltd Property Limited
<	Less than
>	Greater than
®	Registered Trademark
µL	Microlitre
µM	Micromole
3’	Three prime
5’	Five prime
A	Adenine
A_E	Effective number of alleles
AMOVA	Analysis of Molecular Variance
A_N	Number of alleles
AO	Atlantic Ocean
A_R	Allelic Richness
BLAST	Basic Local Alignment Search Tool
bp	Basepair
C	Cytosine
CI	Confidence Interval
CTAB	Cetyltrimethylammonium Bromide [$((C_{16}H_{33})N(CH_3)_3Br]$
dH_2O	Distilled Water
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
EST	Expressed Sequence Tag
F	Forward Primer

FAM	Blue (R100); 5-carboyfluirescein (ABI-fluorescent label)
FB	False Bay Population
F_{CT}	Derivative of Wright's Fixation Index adapted for hierarchical AMOVA (group of populations relative to the total population)
F_{IS}	Wright's Fixation Index (individual relative to the sub-population, equal to the inbreeding coefficient - *f*)
Fr_{NULL}	Null allele frequency
F_{ST}	Wright's Fixation Index (subpopulation relative to the total population)
g	Grams
G	Guanine
H_O	Observed Heterozygosity
H_E	Expected Heterozygosity
HRM	High Resolution Melt
IAM	Infinite Allele Model
IBD	Isolation by Distance
IUCN	International Union for Conservation of Nature
K2P	Kimura 2-parameter model
LD	Linkage Disequilibrium
L_T	Total Length
M	Molar (Moles per Litre)
mg/mL	Milligram per Millilitre
MgCl2	Magnesium Chloride
min	Minutes
ml	Millilitre
MLRA	Marine Living Resource Act
mM	Millimole
MPS	Multiplex Systems (Assays)
MYA	Million Years Ago

NED	Yellow (Tamra) (ABI-fluorescent label)
NJ	Neighbor-Joining
^{o}C	Degrees Celsius
PCR	Polymerase Chain Reaction
PET	Red (ABI-fluorescent label)
$P_{E\text{-}W}$	Ewens-Watterson Probability
PIC	Polymorphic Information Content
P-value	Probability value (as a statistically significant limit)
R	Reverse Primer
s	Seconds
SMM	Stepwise Mutation Model
SNP	Single Nucleotide Polymorphism
spp.	Several Species
SSR	Simple Sequence Repeat
STR	Short Tandem Repeat
T	Thymine
T_A	Annealing Temperature
TAC	Total Allowable Catch
TAE	Total Allowable Effort
Taq	*Thermus aquaticus* DNA polymerase
™	Trademark
U	Units (enzyme)
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
VIC	Green (ABI-fluorescent label)
v/v	Volume per Volume
w/v	Weight per Volume
YBP	Year Before Present

“Plenty sits still. Hunger is a wanderer.”

— Zulu proverb

CHAPTER 1

More than a drop in the ocean: introduction, research aims and objectives, and dissertation outline

1.1. Elasmobranchs: Biodiversity and threats

Elasmobranchs (sharks, skates and rays) are the earliest ancient extant jawed vertebrates – of cartilaginous fishes – belonging to the subclass Elasmobranchii in the class Chondrichthyes (Bigelow and Schroeder 1948; Ebert *et al.* 2013*a*). As elasmobranchs have survived four mass extinction events (Raup and Sepkoski 1982), the lineage is considered as one of the most evolutionarily successful lineages in terms of historical endurance. Most present-day taxa are thought to be derived from Mesozoic forms *e.g.*, *Palaeospinax, Synechodus* (Maisey *et al*. 2004; Grogan and Lund 2004). Increasing fossil evidence indicates the presence of elasmobranchs from the lower Devonian *c*. 400 million years ago, and possibly Silurian *c*. 416 million years ago (Maisey *et al*. 2004). Elasmobranchs became widely distributed during the Age of Fishes (Devonian) and exhibit some of the greatest diversity of morphological and ecological forms in present time (Maisey *et al*. 2004; Grogan and Lund 2004; Adnet and Cappetta 2008). Currently, valid elasmobranch species include nine orders, 34 families, 105 genera and 517 species of sharks; four orders, 27 families, 104 genera and 646 species of batoids *i.e.*, skates and rays (Weigmann 2017).

Previous analyses of biogeographical diversity of chondrichthyan species (including chimaeras) show that there are six biodiversity hotspots in the world. In order of biodiversity ranking these are: Australia, Japan, Indonesia, southern Africa, Central West Atlantic and Taiwan (Carpenter 2002; Last and White 2011; Ebert *et al.* 2013*b*; Ebert and van Hees 2015; **Figure 1.1a** and **1.1b**). However, Weigmann (2016) provides a complete global analysis of the detailed biogeographical diversity of

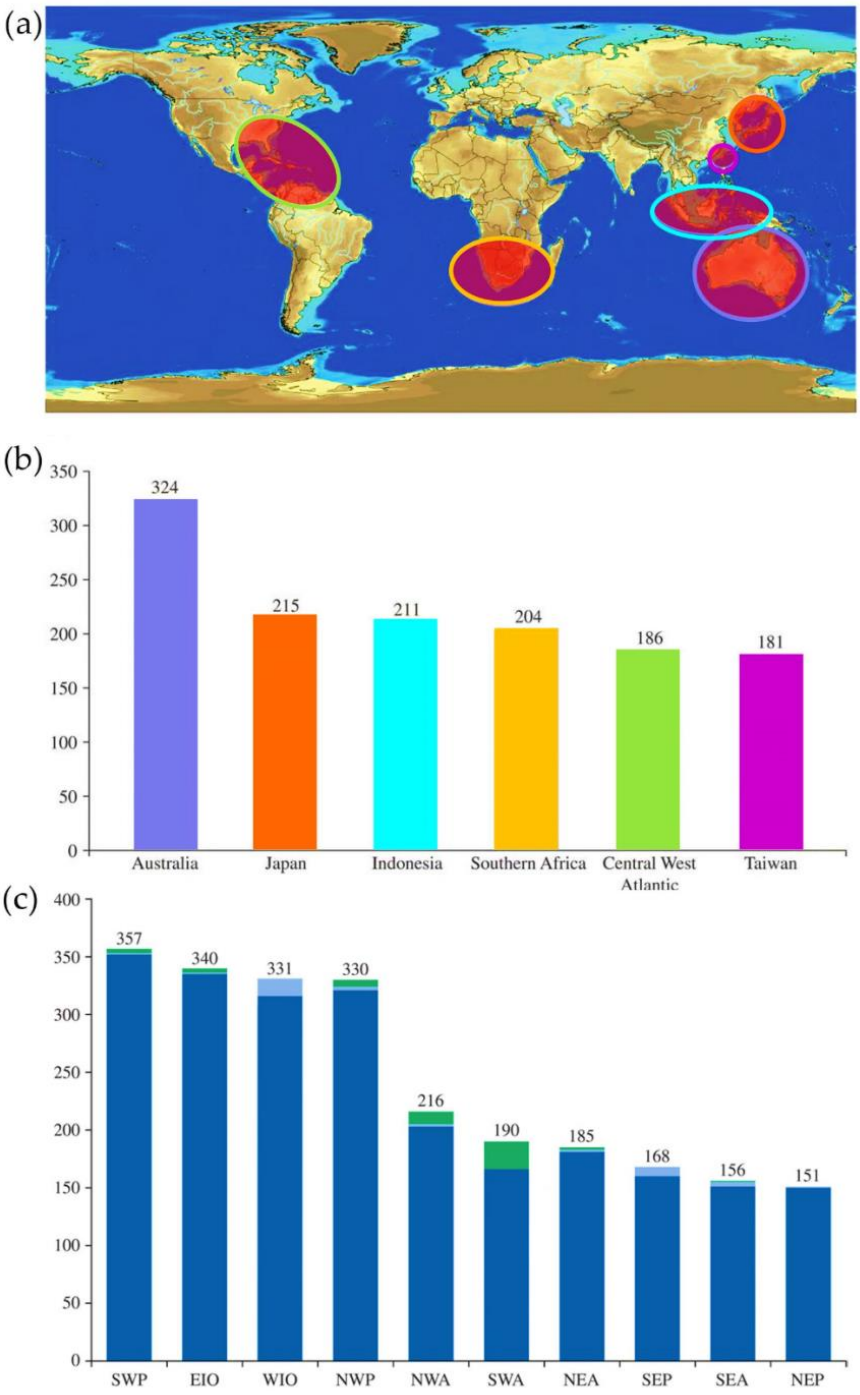

Figure 1.1 Hotspots of chondrichthyan biodiversity. (a) Geographic distributions of previously recognised hotspots, (b) number of chondrichthyan species per hotspots, where entire bars and numbers above the bars indicate the total number of known species per major area and (c) revised biogeographical diversity of chondrichthyans for 10 major areas where the number of questionably occurring species (•) and the number of species found only in fresh and brackish water (•) are indicated. SWP, south-western Pacifc Ocean; EIO, eastern Indian Ocean; WIO, western Indian Ocean; NWP, north-western Pacifc Ocean; NWA, north-western Atlantic Ocean; SWA, south-western Atlantic Ocean; NEA, north-eastern Atlantic Ocean; SEP, south-eastern Pacifc Ocean; SEA, south-eastern Atlantic Ocean; NEP, north-eastern Pacifc Ocean. Modified from Weigmann (2016).

chondrichthyans for 10 major oceanic regions, with standardised area sizes (**Figure 1.1c**). Corroborating earlier annotations (Myers *et al.* 2000; Roberts *et al.* 2002; White and Sommerville 2010; Wafar *et al.* 2011; Last *et al.* 2014), Weigmann (2016) shows that the highest number of biodiversity hotspots are found in the Indo-West Pacific Ocean. However, it is predicted that poorly investigated regions such as the deep western Indian Ocean are host to a large number of undescribed species. More specifically, southern Africa (Angola, Namibia, South Africa and Mozambique) is one of the custodian regions of chondrichthyan biodiversity within the eastern extent of its long and diverse coastline, comprising rocky and sandy shores, kelp forests, estuaries and coral reefs. The southern African chondrichthyan fauna includes 13 orders, 49 families, 111 genera and approximately 204 species, representing nearly 20% of all known chondrichthyans (Ebert and van Hees 2015).

Globally, sharks are facing many challenges leading to global extinctions and population declines (Dulvy *et al*. 2014; Davidson *et al*. 2015). Eighteen percent of the assessed and non-data deficient elasmobranch species are classed as vulnerable, endangered or critically endangered by the International Union for the Conservation of Nature (IUCN) Shark Specialist Group (SSG) (Dulvy *et al*. 2014, 2017). As expected, the majority of these species are coastal elasmobranchs which have a higher risk of exposure to different types of fishing practices – artisanal, recreational and commercial – mostly outside no-take zones (Chapman *et al*. 2005; Da Silva *et al*. 2013; Espinoza *et al*. 2014; Oliver *et al*. 2015). Aside from fishing pressures, the cumulative extinction risk for these species is a combined response to the life-history traits, such as late age of maturity coupled with low fecundity and relatively slow growth rates (Hoenig and Gruber 1990; Smith *et al*. 1998; Cortés 2000; Frisk *et al*. 2001), as well as the general degradation of coastal, estuarine and riverine habitats of coastal sharks (Shepherd and Myers 2005; Dudley and Simpfendorfer 2006). Moreover, a study by Chin *et al*. (2010) indicated the climate-sensitivity of some elasmobranchs using an integrated risk assessment for climate change. In that study, 30 elasmobranch species are recognised as moderately or highly vulnerable to climate change, especially the

rare species including the freshwater whipray *Himantura dalyensis* and the eastern spotted gummy shark *Mustelus walkeri*. Despite these vulnerabilities and the ecological significance of elasmobranchs in marine ecosystems, knowledge on basic biology, population dynamics, and behaviour is for the most part still lacking especially for non-charismatic species.

1.2. Status of elasmobranch fisheries and management

Historically, elasmobranchs have been viewed mostly as unpalatable fish with low product value owing to high levels of urea and the fact that related product such as meat and fins are often dried for non-perishable storage and transport (Bonfil 1994; Clarke *et al*. 2007). As such, elasmobranch fisheries represent a relatively low economic value of *c*. US$1 billion per year contribution to the overall fisheries production of most nations, compared with the teleost fisheries at US$129.8 billion in 2012 (Food and Agriculture Organisation, FAO 2014). Consequently, very limited information has been recorded on the catches and landings of elasmobranchs (Castro *et al*. 1999; Clarke *et al*. 2007) while species-level data required for basic resource assessments are almost non-existent (Baum *et al*. 2003; Lack and Sant 2009; FAO 2014). Globally, elasmobranch fisheries can be categorised as traditional, artisanal, industrial, bather-protection-orientated or recreational (reviewed in Walker 1998). These can be divided into two principle components; directed (targeted) fisheries and non-directed (bycatch) fisheries (FAO 1995, 1999; Clarke *et al*. 2007). More than 90% (617 722 tonnes) of the world's reported elasmobranch catch (especially sharks) is taken by 26 fishing nations including Japan, New Zealand and United States. In the fin trade however, 90% (8 584 tonnes) of the total reported fin exports to Hong Kong is dominated by 23 countries including Australia, Namibia and South Africa (**Figure 1.2**; Dulvy *et al*. 2017). In Dulvy *et al*. (2017) it is further showed that 40% of the reported global shark catch comes from seven major shark fishing nations with the lowest Human Development Indices (a composite statistic of life expectancy, education, and per capita income indicators),

including Indonesia, India, Nigeria, Pakistan, Yemen, Tanzania and Senegal (**Figure 1.2**).

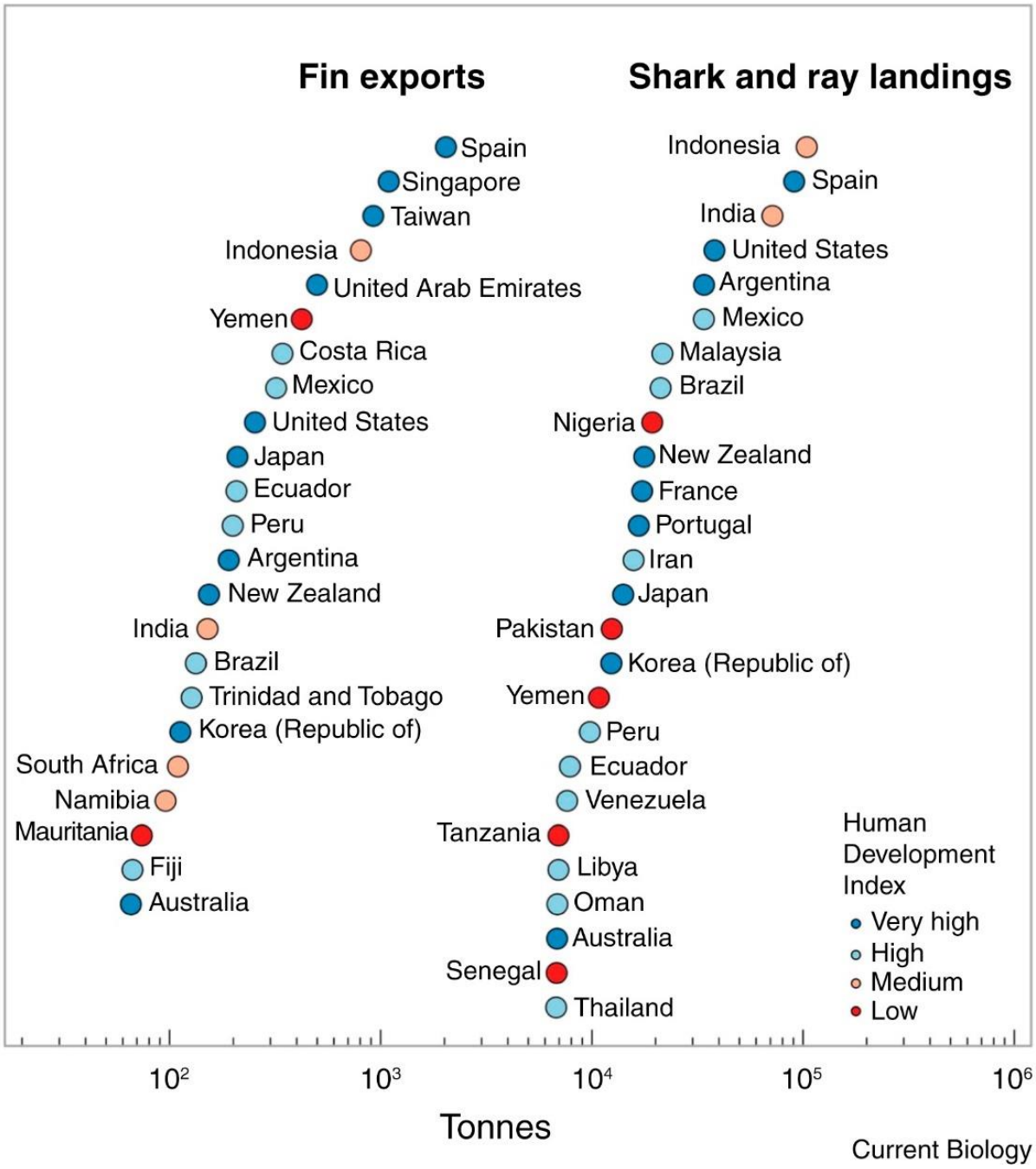

Figure 1.2 Human Development Index, fin trade, and fisheries catches of elasmobranchs reported to the United Nations Food and Agriculture Organization (FAO). Countries shown to the left of each data point are among the least developed with a low or medium Human Development Index category, those labelled to the right are the most developed nations with high or very high Human Development Indices. The unlabelled *y*-axis is the rank order of the named countries. Adapted from Dulvy *et al*. (2017).

Over the years, increasing awareness of the vulnerability of sharks to harvesting and finning (*i.e.*, removal of fins and discarding of the carcass at sea) have contributed to growing concerns on resource sustainability (Friedrich *et al*. 2014; Dulvy *et al*. 2014). Overexploitation of elasmobranchs continues to increase owing to a combination of improved fishing gear and effort as well as the ongoing decline of several teleost fisheries (Stevens *et al*. 2000; Dulvy *et al*. 2008). Consequently, many populations of different oceanic and coastal shark species have undergone rapid declines due to fishing rates which far exceed population rebound rates in many regions (Musick *et al*. 2000; Baum *et al*. 2003, 2004; Dulvy *et al*. 2008). In fact, large coastal areas once abundant with an array of shark species are now depleted of sharks (Ferretti *et al*. 2008; Ward-Paige *et al*. 2010).

Since 1990, attention has been focused on the above-mentioned issues which led to the establishment of an International Plan of Action for Sharks (IPOA-Sharks), including skates, rays, and chimaeras (FAO 1999). The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) has also played a critical role in addressing international exploitation of elasmobranchs (Clarke 2004; Mundy-Taylor and Crook 2013). And, very recently, the Port State Measures Agreement went into force, which aims to address all forms of illegal, undocumented and unreported (IUU) fishing, also for sharks (http://www.fao.org/port-state-measures/background/en/). Conservation efforts have progressed by integrating molecular genetic data into existing fisheries stock-assessment models in order to improve the sustainable harvesting of stocks (Francis and Lyon 2012; Ovenden 2013). In actual fact, Simpfendorfer & Dulvy (2017) provide conclusive evidence showing that in the least 9% of the current global catch of sharks, from at least 33 species investigated, is biologically sustainable. Instead of extreme measures such as bans (*e.g.* Ward-Paige *et al*. 2012), the goal of shark conservation should therefore be directed towards sustainable fisheries management (Simpfendorfer and Dulvy 2017).

Similar to other nations, the Republic of South Africa has dedicated and well-established elasmobranch fisheries including the demersal shark longline fishery and

pelagic longline fishery (reviewed in Da Silva *et al*. 2015). Target species include smoothhounds *Mustelus mustelus*, whitespotted smoothhound *M. palumbes*, soupfin *Galeorhinus galeus*, bronze whaler *Carcharhinus brachyurus*, dusky sharks *Carcharhinus obscurus*, and broadnose sevengill shark *Notorynchus cepedianus* (Da Silva 2007; Da Silva and Bürgener 2007). In addition, four decommercialised species, the leopard catshark *Poroderma pantherinum*, striped catshark *P. africanum*, spotted gully shark *Triakis megalopterus*, and spotted ragged-tooth shark *Carcharias taurus* are occasionally landed, although the trade in the latter is no longer permitted (Da Silva *et al*. 2015). Annual landings of elasmobranch are reported as 277 tonnes, 175 tonnes and 165 tonnes between 2010 and 2012, comprising 14 reported species. In South Africa, shark meat is of little importance to the local market and the bulk of processed shark meat is exported to Australia for the fish and chip trade (Da Silva and Bürgener 2007), while the fins are exported to Asian countries primarily for the fin trade (Fowler 2005; see **Figure 1.2**).

In South Africa, elasmobranchs are managed and regulated under the *Marine Living Resources Act 18 of 1998* (MLRA; RSA 1998) by the fisheries management branch of Department of Agriculture, Forestry and Fisheries (DAFF 2013). Coastal Marine Protected Areas (MPAs), *e.g.* the Langebaan Lagoon MPA and De Hoop MPA, have been established to offer partial protection to various coastal shark species, such as ragged-tooth sharks, cow sharks, smoothhounds, catsharks and juvenile requiem sharks (Griffiths *et al*. 2010). Fisheries management also monitors entry into any commercial fishery by a rights allocation process. The latter is based on scientific recommendations in limiting the number of vessels, crew and Total Allowable Catch (TAC) or Total Allowable Effort (TAE) for target species, in addition to precautionary catch limits for bycatch species (DAFF 2013). Also, South Africa implements shark management actions via a Nation Plan of Action for Sharks established in 2001 (NPOA-Sharks) (DAFF 2013).

1.3. Insights from molecular studies on elasmobranchs

Chondrichthyans (*i.e.*, elasmobranchs and chimaeras) diverged from a common ancestor of bony vertebrates (Osteichthyes: ray-finned fishes, coelacanths, lungfishes, and tetrapods) in the early Silurian (Benton *et al*. 2009). The phylogenetic position of chondrichthyans as a basal clade to bony vertebrates on the tree of life (Douady *et al*. 2003; Maisey *et al*. 2004; Mallatt and Winchell 2007; Inoue *et al*. 2010), have made these fish a critical reference in the understanding of vertebrate genome evolution (Venkatesh *et al*. 2007; Yu *et al*. 2008; Larsson *et al*. 2009; Ravi *et al*. 2009). In other words, chondrichthyans are a valuable outgroup in comparative studies of vertebrates. Chondrichthyans are relics of an ancient world which have endured extreme ecological change and numerous mass extinction events, yet today these fish are faced with variable risks of extinction owing to anthropogenic threats (Friedrich *et al*. 2014; Dulvy *et al*. 2008, 2014). This has motivated genetic studies and the use of molecular tools to address fundamental ecological and evolutionary questions to assist fisheries management and conservation (reviewed in Dudgeon *et al.* 2012; Portnoy and Heist 2012; Bester-van der Merwe and Gledhill 2015).

Molecular genetic analysis provides a platform for conservation biologists to critically evaluate additional factors that may be causing declines (*e.g.* population or genetic bottlenecks). Also, the use of genetic data allows for assessing the two key elements in delineating management units, population structure and phylogeography (Moritz 1994; Palsbøll *et al*. 2007). Until recently, the highly migratory nature of many elasmobranch species and difficulties associated with field observation of behaviour has hindered the understanding population and reproductive biology of these animals. Molecular techniques also offer an alternative and innovative way of addressing classical ecological questions while providing novel insights into behaviour and biology of species.

Species identification in several elasmobranch genera (e.g. *Haploblepharus*, *Mustelus*) based only on alpha taxonomy can be a difficult process due to the highly

conservative nature of species morphology within genera (Gardner and Ward 2002; Human 2007; Farrell *et al.* 2009; Marino *et al.* 2017). This not only confounds defining species boundaries (Boomer *et al*. 2012) but also negatively impacts the collection of species-specific catch data in fisheries operations (Da Silva *et al*. 2015). This led to the development of molecular species identification assays to resolve species misidentification issues as accurate identification of species can be seen as the first step for recording and quantifying biodiversity.

Genetic surveys have mostly advanced our knowledge on the historical and contemporary processes driving elasmobranch distribution patterns (Vignaud *et al*. 2013; O'Brien *et al*. 2013; Sandoval-Castillo and Beheregaray 2015; Dudgeon and Ovenden 2015). Genetics research enables the defining of reproductively isolated stocks, characterising genetic variability and assessing the direction and strength of gene flow between populations across smaller (Mendonça *et al*. 2013; Mourier *et al*. 2013; Bitalo *et al*. 2015) and larger spatial scales (Benavides *et al*. 2011*a*, *b*; Karl *et al*. 2011; Vignaud *et al*. 2014; Veríssimo *et al*. 2017). Based on a number of genetic studies, it is now recognised that pelagic and highly mobile species harbour little (if any) genetic heterogeneity (*e.g*., Boomer 2013; Da Silva Ferrette *et al*. 2015; Verríssimo *et al*. 2017) while coastal and more sedentary species exhibit relatively high genetic heterogeneity (*e.g*., Barker *et al*. 2015; Bitalo *et al*. 2015; Chabot *et al*. 2015). The inferred population genetic structure in elasmobranchs is not only influenced by the interactions between evolutionary forces (gene flow and genetic drift) but also by life history traits (genetic mating system, philopatry) and seascape features such as hydrodynamic, thermal and other ecophysiological boundaries (Chabot and Allen 2009; Veríssimo *et al*. 2010*a*; Karl *et al*. 2011; Karl *et al*. 2012; Tillett *et al*. 2012; Vignaud *et al*. 2014; Bitalo *et al*. 2015).

Knowledge on the mating (copulating) strategy of elasmobranchs remains poorly understood due to the lack of direct behavioural observations during mating in the wild. However, molecular techniques enable for example the testing for the presence or absence of multiple paternity (where a single litter is sired by multiple males).

Multiple paternity studies indicate complex genetic mating systems with considerable interspecific variability in several elasmobranchs (Chapman *et al*. 2003, 2004; Dibattista *et al*. 2008; Daly-Engel *et al.* 2010; Verissimo *et al.* 2010*b*; Chapman *et al*. 2013; Rossouw *et al*. 2016). The presence of multiple paternity is widely accepted as indicative of a polyandrous mating system (where females mate with multiple males in a single breeding season), although it is also possible that it is as a result of long-term sperm storage (across multiple breeding seasons) (Farrell *et al*., 2014; Marino *et al*., 2015). Moreover, recent studies show that the frequency of multiple paternity can vary across geographically separated populations of the same species (Daly-Engel *et al*. 2007; Portnoy *et al*. 2007; Boomer *et al.* 2013; Chabot and Haggin 2014; Marino *et al.* 2015; Rossouw *et al*. 2016).

It is evident that genetic surveys are an integral component of elasmobranch research and will continue to expand the knowledge base of the biology and ecology of these marine species. Furthermore, conservation genetics is currently undergoing a transition to conservation genomics. It is anticipated that the on-going developments in sequencing technologies will enable identification of genes important for local adaptation or those that play a critical role in mediating key life history traits such as dispersal strategies (residency versus migratory).

1.4. Dissertation research aims and outline

The overall aim of this study is to advance the limited available knowledge on the shark genus *Mustelus* with particular reference to southern African species of the genus, within the broader scope of delineating species population dynamics and biology for management and conservation. The study implements a multidisciplinary approach (molecular, next-generation sequencing, morphometrics and histology) to disentangle the evolutionary origin of the shark genus *Mustelus* in southern Africa, develop genetic resources such as microsatellites markers, resolve species identification issues within the genus, as well as investigate the reproductive biology

of the genus. A critical and comprehensive literature survey was completed and, through international and local collaborations exhaustive sampling was successfully carried out to address the following questions:

Chapter 2: What are the key research gaps and challenges that will, if addressed in an appropriate and timely manner, significantly advance our knowledge on the evolution, molecular ecology and conservation of species of the shark genus *Mustelus*?

Here, a synthesis of literature spanning three decades is provided to identify key research gaps as well as to address molecular taxonomy, the discordance between nuclear and mitochondrial DNA data sets, genetic aspects of breeding behaviour and corresponding evolutionary, and conservation implications.

Chapter 3: 1) How many colonization events resulted in the present southern African *Mustelus* species and from where did they originate? 2) Did any colonization event lead to a species radiation? 3) What is the origin of black spots in the genus?

Species of *Mustelus* display a peculiar distributional pattern with a high degree of sub-regional endemism. This observed pattern of distribution and high levels of endemism in *Mustelus* is postulated to have occurred due to the interaction of different oceanographic phenomena with the migration ability of these species, thereby causing sudden subsidence and resulting in these species being restricted to smaller geographical ranges. Examining the evolutionary origin of species of *Mustelus* using a historical biogeographic approach is anticipated to shed light on the process that led to the observed geographic patterns of species distributions in the present day.

Chapter 4: Are the newly developed microsatellite loci suitable to infer patterns of genetic diversity and differentiation in houndsharks (family Triakidae)?

Knowledge on the distribution of genetic diversity and population structure is essential for appropriate allocation of management units for species. Here, a newly developed panel of 11 microsatellite loci for the common smoothhound shark

Mustelus mustelus was characterised and tested for cross-species amplification in six triakid species. These microsatellites loci were employed to assess the distribution of genetic variation across populations of four co-distributed sharks sampled along the South African coast. This presented an ideal opportunity to test whether the interplay of oceanographic features and life history traits are the drivers of population subdivision in these sharks, and make inferences on the pragmatism of a multispecies management approach.

Chapter 5: Are the newly developed single nucleotide polymorphisms (SNPs) suitable to infer patterns of genetic diversity and differentiation in species of *Mustelus*?

Development of genetic tools in these sharks has been hampered in part by the lack of a reference genome sequence, and this continues to present a huge challenge for molecular ecology studies. In non-model species, the development of molecular markers is now circumvented using high throughput sequencing approaches which do not require a reference genome *e.g.*, reduced representation sequencing approaches. Here, SNP loci were developed and a subset validated to augment the existing molecular marker repository for the common smoothhound shark. The successfully validated subset SNP panel was also tested for cross-species amplification in a closely related and sympatric species of *Mustelus*, the whitespotted smoothhound shark *M. palumbes*.

Chapter 6: 1) Can microsatellite loci differentiate between species of *Mustelus* and morphologically similar houndsharks? 2) Can a high-resolution melting analysis be used for identifying six houndshark species? 3) Do dermal denticles possess species-specific diagnostic characters? 4) What morphometric measurements are suitable for identifying headed and gutted (dressed) sharks?

To bridge the gap between conservation genetics, fisheries management and law enforcement, it is prudent to evaluate and provide efficient methods for species-level

identification of sharks. Here, three molecular screening methods were optimised and evaluated for the identification of six houndsharks (*Galeorhinus galeus*, *Mustelus mosis*, *M. mustelus*, *M. palumbes*, *Scylliogaleus quecketti* and *Triakis megalopterus*). In addition, the dermal denticles were analysed via light microscopy for their ability to discriminate between species. Finally, several morphometric measurements of dressed fish were assessed for its suitability in differentiating *Mustelus mustelus* from *M. palumbes*.

Chapter 7: 1) Does *M. mustelus* have the ability to store sperm as evidenced by histologic approaches? If so, what is the potential effect on multiple paternity and polyandry? 2) Does multiple paternity exist within a litter of the common smoothhound sampled along the south–west coast of South Africa?

Knowledge on the reproductive strategies is also integral for species management and conservation. Here, sperm storage and multiple paternity in commercially important smoothhound sharks are assessed, including the common smoothhound and whitespotted smoothhound. This will contribute to the growing body of knowledge regarding the genetic and behavioural mating strategy of smoothhound sharks in South Africa.

Chapter 8: What have we learnt and what could we learn in the future?

This chapter discusses the main findings of this dissertation and the implications thereof in filling the knowledge gaps noted in the literature survey conducted in Chapter 2. The chapter then examines possible future scientific directions that will advance the available knowledge base on smoothhounds.

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CHAPTER 2

Molecular research on the systematically challenging smoothhound genus *Mustelus*: a synthesis of the past 30 years

Abstract

The species-rich shark genus *Mustelus*, or smoothhounds (smoothhound sharks), is one of the most bio-economically important groups of elasmobranchs in the world's oceans. Despite the commercial value of *Mustelus*, its systematics remains largely unresolved and there is no global review or synthesis of knowledge about the conservation status and conservation genetics of smoothhounds across all oceanic regions. Here, published studies as well as grey literature were accessed to gain insight into the biogeographic, ecological and behavioural factors that shape genetic diversity in smoothhounds, as well as to identify critical knowledge gaps. From a series of molecular phylogenetics studies it can be inferred that the shark genus *Mustelus* is paraphyletic and that the aplacental species of *Mustelus* secondarily evolved from placental species of the genus. The use of both genetic and morphological characteristics continues to guide the development of practical field identification keys for co-occurring smoothhound sharks. Furthermore, it is evident that different smoothhound species exhibit unique gene flow patterns, suggesting that species-level conservation approaches would be most appropriate. Finally, molecular studies have advanced our understanding of smoothhound biology (including reproductive traits), ecology and evolution, while many gaps in knowledge remain.

2.1. Introduction

Globally, shark species are facing many challenges leading to population declines and possibly also to unknown local extinctions (Dulvy *et al.* 2014; Davidson *et al.* 2015). The complex life-history and ecological features of shark species render them vulnerable to anthropogenic impacts such as harvesting, habitat loss, and habitat degradation (Smith *et al.* 1998; Baum *et al.* 2003; Shepherd and Myers 2005; Dudley and Simpfendorfer 2006; Chin *et al.* 2010). A plethora of studies stress the need of instigating genetic studies to assist in addressing critical fisheries management and conservation issues while providing novel insights into evolution, biology and behaviour of shark species (reviewed in Dudgeon *et al.* 2012; Portnoy and Heist 2012; Bester-van der Merwe and Gledhill 2015). Molecular genetic analysis provides a toolbox for conservation biologists to identify additional factors that may cause population declines (*e.g.*, population bottlenecks, outbreeding depression) and obtain information on population parameters (structure, dispersal and effective population size) and mating behaviour.

Molecular surveys in sharks were formally inaugurated by Smith (1986), who sought to explain the low levels of genetic variation (relative to other marine fishes) he observed in a coastal shark, *Mustelus lenticulatus*, using allozymes (enzymatic proteins that have allelic variation at a single locus). In the former study, six additional species (*Centroselachus crepidater* then *Centroscymnus crepidater*, *Deania calcea*, *Etmopterus baxteri*, *Galeorhinus galeus* then *G. australis*, *Prionace glauca* and *Squalus acanthias*) were included to determine whether low genetic variation was characteristic in sharks. Smith (1986) found low allozyme variation – measured by mean heterozygosity and the percentage of polymorphic loci – across all the study species. In marine teleosts, Smith & Fujio (1982) previously proposed that habitat specialists had greater genetic variability than habitat generalists. Smith (1986) then concluded that the low allozyme variation observed in sharks was attributed to sharks being

habitat generalists supporting the 'habitat specialist-generalist model' of Smith & Fujio (1982). However, aside from the known limitations of allozymes, it is now widely accepted that sharks have lower genetic diversity than other vertebrates, possibly because of low rates of molecular evolution and long generation time (Martin *et al.* 1992; Martin 1995; Heist *et al.* 2003; Karl *et al.* 2011; Tavares *et al.* 2013). Some 30 years have elapsed since researchers undertook molecular investigations in sharks; however, in the current review the focus is primarily on the smoothhound shark genus *Mustelus*.

The cosmopolitan genus *Mustelus* (Linck, 1790) represents a fascinating genus of chondrichthyans (cartilaginous fishes) in the Triakidae family belonging to the order Carcharhiniformes (ground sharks) with high levels of regional endemicity. Species of *Mustelus*, or smoothhounds (also called smooth dogfish, gummy sharks or palombos), are small to medium-sized (57 – 185 cm total length) demersal sharks abundant in temperate and tropical nearshore habitats, from shallow waters to a maximum depth of about 1463 m (Compagno 1984; Heemstra 1997; Weigmann 2016). Smoothhounds are ecologically important mesopredators (mid-level predators) and often exposed to different types of fishing practices including artisanal, recreational and commercial fisheries (Heemstra 1997; Gardner and Ward 2002). Smoothhounds are all viviparous (live-bearing), with two discrete modes of reproduction based on fetal nutrition: placental viviparity, wherein initial yolk-sac fetal nutrition is later augmented by additional maternal nutrients via a yolk-sac placenta; and yolk-sac viviparity, wherein the yolk-sac is the principal source of fetal nutrition throughout development to parturition (Heemstra 1973; Compagno 1984). However, both the studies of Storrie *et al.* (2008) and Farrell *et al.* (2010) revealed that white spotted smoothhounds (*Mustelus antarcticus* and *M. asterias*) previously thought to be yolk-sac viviparous are in fact minimal (mucoid) histotrophs. Mucoid histotrophy is a viviparous mode of reproduction whereby initial yolk-sac fetal nutrition is later augmented by additional maternal nutrients in the form of mucous produced by the uterus throughout development to parturition (Hamlett *et al.* 2005; Musick and Ellis

2005). Here, the term 'aplacental' viviparity is reserved to include both yolk-sac viviparity and mucoid histotrophs given that both these reproduction modes lack a placenta.

The smoothhound shark genus *Mustelus* is one of the most problematic elasmobranch genera with respect to taxonomy and systematics (Heemstra 1973; Heemstra 1997; López *et al.* 2006; Boomer *et al.* 2012; Naylor *et al.* 2012). Identifying species complexes in *Mustelus* based on alpha taxonomy can be a difficult process as many of the morphological, morphometric and meristics characters are highly variable within species with considerable overlap between species (Heemstra 1973; Compagno 1984; Heemstra 1997; Gardner and Ward 2002; Rosa and Gadig 2011). Moreover, species of the genus *Mustelus* may be confused with other triakid species in the genera *Iago* and *Triakis* (Kato 1968; Compagno 1970, 1973; Heemstra 1973; Akhilesh *et al.* 2014; Weigmann 2016). For example, Heemstra (1973) in his revision of *Mustelus* considered the formerly described *Mustelus megalopterus* (Smith 1839) or *M. nigropunctatus* (a junior synonym of *M. megalopterus*, Smith 1952) as a valid species of *Triakis* (Müller and Henle 1838). Ebert *et al.* (2013) on the other hand reassigned *M. mangalorensis* (Cubelio *et al.* 2011) to the genus *Iago* (Compagno and Springer 1971). Currently, it is uncertain whether this species is conspecific with *Iago omanensis* or a valid species in the *I. omanensis* complex since the holotype could not be located (Akhilesh *et al.* 2014; Weigmann 2016). Consequently, the genus *Mustelus* previously thought to comprise 31 extant species (Naylor *et al.* 2012), presently has 27 valid extant species, **Table 1.1** (Ebert *et al.* 2013; Weigmann 2016). Distinguishing between valid species of *Mustelus* involves recognising subtle differences in morphometric features, such as, position of fins, internarial distance, denticle and tooth configurations, buccopharyngeal denticle patterns, vertebral numbers and labial furrow size (Heemstra 1973; Heemstra 1997; Rosa and Gadig 2011). The state of these traits may not be accessible, or easily recognised by non-experts when identifying recognised species of *Mustelus* in the field. In fact, both scientists and fisheries managers often experience difficulties in distinguishing between species of *Mustelus* in the field based

on traditional morphological keys (Gardner and Ward 2002; Da Silva and Bürgener 2007; Akhilesh *et al.* 2014; Giresi *et al.* 2015; Marino *et al.* 2017). Revised identification keys that permit more reliable identification of recognised co-occurring (sympatric) species of *Mustelus* in the field are becoming more available (Giresi *et al.* 2012*a*; Giresi *et al.* 2015; Marino *et al.* 2017).

The International Union for the Conservation of Nature (IUCN) Shark Specialist Group (SSG) has assessed all species of *Mustelus* (**Table 2.1**). At present, one species (*M. fasciatus*) is classed as Critically Endangered, one species (*M. schmitti*) as Endangered, two as Vulnerable (*M. mustelus* and *M. whitneyi*), two as Near Threatened (*M. canis* and *M. mento*), nine as Least Concern and 12 as Data Deficient (Francis 2003; Leandro 2004; Hozbor *et al.* 2004; Conrath 2005; Faria and Furtado 2006; Leandro and Caldas 2006; Smale 2006; Massa *et al.* 2006; Carlisle 2006; Romero 2007; Romero *et al.* 2007; Cronin 2009; Serena *et al.* 2009*a*, *b*, *c*; Quaranta and Ebert 2009; Tanaka *et al.* 2009; Valenti 2009; Jones *et al.* 2009; White 2009; McAuley 2011; McAuley and Kyne 2011; Pérez-Jiménez *et al.* 2015; Walker 2016; Pérez-Jiménez *et al.* 2016: Pérez-Jiménez 2016; Kyne and Rigby 2016). While for a few species (*M. antarcticus*, *M. lenticulatus* and *M. ravidus*) population trends have been reported as stable or increasing (Francis 2003; McAuley 2011; Walker 2016), population trends are reported as decreasing for several other species (*M. fasciatus*, *M. mento*, *M. mustelus*, *M. schmitti* and *M. whitneyi*) (Hozbor *et al.* 2004; Massa *et al.* 2006; Romero 2007; Romero *et al.* 2007; Serena *et al.* 2009*b*). The current conservation status of species of *Mustelus* is, however, difficult to assess as numerous assessments are more than a decade old and needs updating by the IUCN SSG. Uncertainty surrounding the conservation status of smoothhounds is further confounded by the gaps in knowledge of the taxonomic status of the shark genus *Mustelus* (Heemstra 1997; Da Silva and Bürgener 2007; Farrell *et al.* 2009; Barbuto *et al.* 2010; Giresi *et al.* 2012*a*; Pérez-Jiménez *et al.* 2013).

This chapter provides a synthesis of the primary (published) and grey (unpublished) literature of available genetic data on aspects of phylogeny, population genetics, phylogeography, mating behaviour and hybridisation. More specifically, the

chapter reports on molecular taxonomy, the discordance between nuclear DNA and mitochondrial DNA data sets, genetic aspects of breeding behaviour and corresponding evolutionary, and conservation implications. Finally, the relevance of the emerging field of conservation genomics is explored, and the potential of conservation genomics to understand broader evolutionary and ecological questions are highlighted.

2.2. Materials and methods

To retrieve a representative collection of research articles, a simple search of the Google Scholar, Shark-References (Pollerspöck and Straube 2017), Scopus and Thompson Reuters' Web of ScienceTM Core Collection using key words and title searches was conducted. Key words were combined using the Boolean operators 'OR' and 'AND'. An asterisk (*) was also used which acts as a 'wildcard' that represents any group of characters, including no character, while a dollar sign ($) represents zero or one character. The following search strings were used "*Mustelus** OR smooth$hound", "(*Mustelus** OR smooth$hound*) AND (genetics* OR molecular* OR paternity* OR barcoding*)", "(*Mustelus** OR smooth$hound*) AND (tagging* OR tracking* OR movement*)". In addition, works cited based on papers identified through electronic database searches were also included. We then filtered query matches by content and only those documents labelled as (research) 'articles', thesis and dissertation using molecular approaches were included. Remaining matches were counted and separated into five categories based on their content. The five categories are: phylogeny, molecular taxonomy, molecular markers, population genetics, phylogeography and genetic mating system.

Table 2.1 Smoothhound shark species of the world including their common names, geographic distributions, presence or absence of white spots and reproductive mode, as well as the current conservation status derived from the International Union for the Conservation of Nature (IUCN) Red List, where DD = Data Deficient, NT = Near Threatened, VU = Vulnerable, EN = Endangered, including the year of assessment. A "?" indicates that reproductive mode is unknown. WIO, western Indian Ocean; EIO, eastern Indian Ocean; SWP, south-western Pacific Ocean; NWP, north-western Pacific Ocean; NEP, north-eastern Pacific Ocean; SEP, south-eastern Pacific Ocean; SWA, south-western Atlantic Ocean; NWA, north-western Atlantic Ocean; NEA, north-eastern Atlantic Ocean including the Mediterranean Sea and Black Sea; SEA, south-eastern Atlantic Ocean; WIO (Red Sea), species endemic to the Red Sea; NEA (Mediterranean), species endemic to the Mediterranean Sea.

Species	Species authority	Common name	White spots	Placental	Distribution	IUCN status
Mustelus albipinnis (or *Mustelus hacat*, a junior synonym of *M. albipinnis*)	Castro-Aguirre Atuna-Mendiola González-Acosta and de la Cruz-Agüero 2005	White-margin fin houndshark	No	Yes	NEP, SEP	DD-2007
Mustelus antarcticus	Günther 1870	Australian smooth(-)hound	Yes	No	EIO, SWP	LC-2015
Mustelus asterias	Cloquet 1819	Starry smooth-hound	Yes	No	NEA	LC-2006
Mustelus californicus	Gill 1864	Grey smooth(-)hound	No	Yes	NEP, SEP	LC-2014
Mustelus canis	Mitchill 1815	Dusky smooth(-)hound	No	Yes	SWA, NWA	NT-2005
Mustelus dorsalis	Gill 1864	Sharptooth smooth(-)hound	No	Yes	NEP, SEP	DD-2004
Mustelus fasciatus	Garman 1913	Striped smooth(-)hound	No	Yes	SWA	CE-2004
Mustelus griseus	Pietschmann 1908	Spotless smooth(-)hound	No	Yes	NWP	DD-2007
Mustelus henlei	Gill 1863	Brown smooth(-)hound	No	Yes	NEP, SEP	LC-2014
Mustelus higmani	Springer and Lowe 1963	Smalleye smooth(-)hound	No	Yes	SWA, NWA	LC-2006
Mustelus lenticulatus	Phillipps 1932	Spotted (estuary) smooth(-)hound/Rig	Yes	No	SWP	LC-2003

Table 2.1 Continued.

Species	Species authority	Common name	White spots	Placental	Distribution	IUCN status
Mustelus lunulatus	Jordan and Gilbert 1882	Sicklefin smooth(-)hound	No	Yes	NEP	LC-2015
Mustelus manazo	Bleeker 1855	Starspotted smooth(-)hound	Yes	No	WIO, NWP	DD-2007
Mustelus mento	Cope 1877	Speckled smooth(-)hound	Yes	No	SEP, SWA	NT-2007
Mustelus minicanis	Heemstra 1997	Venezuelan dwarf smooth(-)hound	No	Yes	NWA	DD-2006
Mustelus mosis	Hemprich and Ehrenberg 1899	Arabian-/hardnose smooth(-)hound/	No	Yes	WIO, EIO	DD-2008
Mustelus mustelus	Linnaeus 1758	Smooth(-)hound/common-/Blackspotted smooth(-)hound	No	Yes	WIO, NEA, SEA	VU-2004
Mustelus norrisi	Springer 1939	Narrowfin-/Florida smooth(-)hound	No	Yes	SWA, NWA	DD-2008
Mustelus palumbes	Smith 1957	Whitespot(ted) smooth(-)hound	Yes	No	WIO, SEA	DD-2006
Mustelus punctulatus	Risso 1827	Blackspot(ted) smooth(-)hound	No	Yes	NEA	DD-2003
Mustelus ravidus	White and Last 2006	Australian grey smooth(-)hound	No	?	EIO, SWP	LC-2003
Mustelus schmitti	Springer 1939	Patagonian/narrownose smooth(-)hound	Yes	No	SWA	EN-2006
Mustelus sinusmexicanus	Heemstra 1997	Gulf of Mexico smooth(-)hound	No	Yes	NWA	DD-2006
Mustelus stevensi	White and Last 2008	Western (white-)spotted gummy shark	Yes	?	EIO	LC-2011
Mustelus walkeri	White and Last 2008	Eastern spotted gummy shark	Yes	?	SWP	DD-2015
Mustelus whitneyi	Chirichigno 1973	Humpback smooth(-)hound	No	Yes	SEP	VU-2007
Mustelus widodoi	White and Last 2006	White(-)fin smooth(-)hound	No	?	EIO	DD-2008

2.3. Results

The literature search yielded 33 studies between 1988 and 2017 (see Appendix). These studies covered molecular phylogenetics, molecular species identification, genetic mating systems, population genetics and phylogeography. Combining data across these literature searches brought together insights in the areas of 1) integrative taxonomy, 2) comparative population structure and phylogeography, and 3) reproductive behaviour and multiple paternity. Overall, this enhanced our knowledge on molecular species identification and several aspects regarding the biology and ecology of smoothhound sharks, including hierarchical population structure and sex-biased gene flow, geographic variation in the rate of multiple paternity as well as the occurrence of hybridisation with potential adaptive trait introgression and evolutionary radiations.

2.4. Discussion

2.4.1. Molecular systematics and integrative taxonomy of Mustelus

Molecular phylogenetic studies of the genus *Mustelus*, based on four protein-coding gene sequences and control region, provide compelling evidence that the genus is paraphyletic and constitute a single monophyletic group when *Scylliogaleus quecketti* and *Triakis megalopterus* are included as shown in **Figure 2.1** (López *et al.* 2006; Boomer *et al.* 2012; Naylor *et al.* 2012). When considering the whole mitochondrial genome comparisons and a more comprehensive taxon sampling of members of *Mustelus*, the species *S. quecketti* and *T. megalopterus* remain within the genus *Mustelus* (Naylor G, perscomm). Interestingly, given the presence of two alternate modes of reproduction within *Mustelus* (placental and aplacental viviparity), several authors advocated for the division of *Mustelus* based on reproductive mode to gain insight into the evolution of the mode of reproduction in the genus. This was however confounded by the fact, that at the time, the mode of reproduction for numerous

species was not yet known (Compagno 1984). More recently, based on phylogenetic evidence the division of the shark genus *Mustelus* based on the mode of reproduction was indeed reasonable. The studies by López *et al.* (2006) and Boomer *et al.* (2012) proposed that *Mustelus* species could be divided into two clades representing different modes of reproduction (placental *vs.* aplacental species) and that these clades are coupled with the presence or absence of white spots on the dorsal surface (**Table 2.1**, **Figure 2.1**).

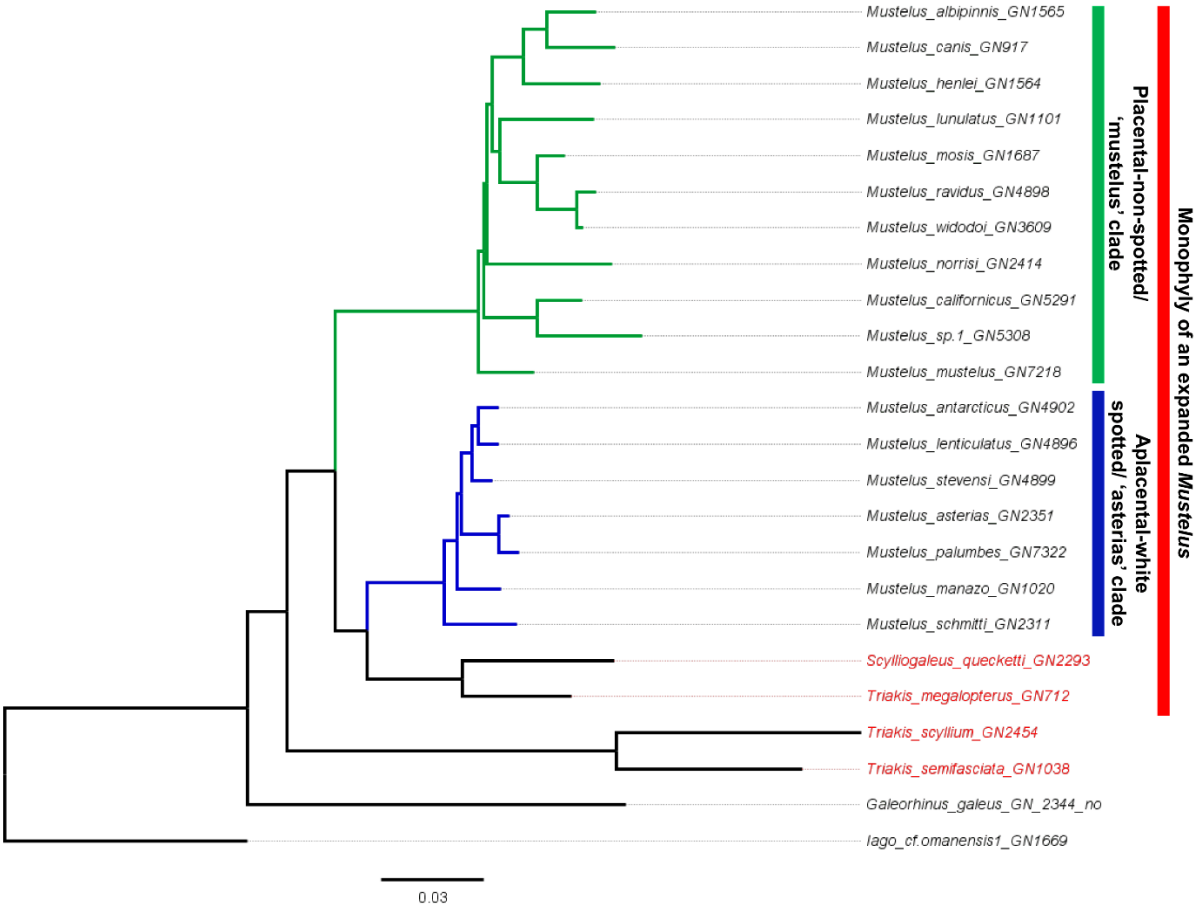

Figure 2.1 Phylogeny of smoothhounds (*Mustelus*) inferred from mitochondrial NADH dehydrogenase subunit 2 modified from Naylor *et al.* (2012). Green and blue labelled branches denote the placental-non-spotted (Boomer *et al.* 2012) or '*Mustelus*' (Lopez *et al.* 2006) clade and aplacental-white spotted (Boomer *et al.* 2012) or 'asterias' (Lopez *et al.* 2006) clade. The red bar denotes the monophyly of an expanded *Mustelus* (including *T. megalopterus* and *S. quecketti*) and highlights the paraphyly of *Triakis*.

Furthermore, this suggested that the phylogenetic coupling of the mode of reproduction with the presence or absence of white spots could in future be valuable for determining the reproductive mode of *Mustelus* species in which the mode of

reproduction has yet to be determined (Boomer *et al.* 2012). This distinction however requires careful revision given that *M. punctulatus*, and to a limited extent *M. mustelus*, have sparse black spots or blotches on their dorsal surface instead of white spots (Farrell *et al.* 2009; Da Silva and Bürgener 2007; Marino *et al.* 2014). The phylogenetic placement of *M. punctulatus* within the *Mustelus* complex also needs to be investigated further (sensu Vélez-Zuazo and Agnarsson 2011). Vélez-Zuazo and Agnarsson (2011) obtained sequence data of four mitochondrial (COI, NADH-2, Cytb, 16S) and one nuclear gene (Rag-1) from GenBank and from the Barcoding of Life Project (www.barcodinglife.org). There is a general misrepresentation of deposited sequences of *M. punctulatus* (mistaken for *M. mustelus*) in these databases owing to previous cases of morphological misidentification (Moftah *et al.* 2011; Marino *et al.* 2014, 2017).

Within the shark genus *Mustelus*, all the aforementioned phylogenetic studies revealed relatively low levels of interspecific genetic divergence, especially within the 'aplacental' clade (**Figure 2.1**). This was indicative of recent divergence and provided two lessons about the impacts of evolution on the genus, the evolution of the two alternative reproductive modes and the possible occurrence of hybridisation within *Mustelus*. Interestingly, past studies of the evolution of reproductive modes in modern elasmobranchs have predicted on oviparity (the laying of eggs) to represent the ancestral reproductive state. However, several authorities refuted this and argued that yolk-sac viviparity is the ancestral state (Musick and Ellis 2005) as previously shown in other vertebrates such as mammals, reptiles, amphibians and fishes (Blackburn 1992; Grogan and Lund 2011; Blackburn 2015). Several molecular phylogenetics studies provided evidence that aplacental viviparous species of *Mustelus* diverged from the placental viviparous species and that aplacental species in fact secondarily evolved from placental species (López *et al.* 2006; Boomer *et al.* 2012; Naylor *et al.* 2012). In other words, the evolutionary reversal from placental to aplacental viviparity in *Mustelus* indicates a secondary loss of placenta. The loss of placenta has recently been reported in a carcharhinid species, the tiger shark (*Galeocerdo cuvier*) by Swift *et al.* (2016) using a phylotranscriptomics approach.

Furthermore, it is known that hybridisation can occur between sympatric and/or allopatric species with similar reproductive modes and overlapping distributions, or when incipient species come into contact following range expansion (Wiley and Lieberman 2011; Morgan *et al.* 2012). In smoothhounds, evidence for this was found by Di Francesco (2011) and recently also reported by Marino *et al.* (2015), who confirmed the existence of interspecific hybrids between the co-occurring placental viviparous smoothhounds, *M. mustelus* and *M. punctulatus*, in the Adriatic Sea using microsatellites and the mitochondrial COI gene. Similarly, Boomer *et al.* (2012) identified a possible hybrid between two allopatric aplacental smoothhounds, *M. antarcticus* from southern Australia and *M. lenticulatus* from New Zealand using microsatellites and the mitochondrial NADH dehydrogenase subunit 4. These observations suggest the possible existence of so-called parapatric species (species that form a hybrid zone in contiguous or narrow overlapping areas; Wiley and Lieberman 2011) and highlights the potential errors associated with basing species delimitation on a small and potentially unrepresentative dataset (Boomer *et al.* 2012; Marino *et al.* 2017). Given the morphological similarities and hybridisation noted among smoothhounds, it appears that occasional adaptive introgression and evolutionary radiations may have been important for the high speciation rates observed in the genus, especially in the central Indo-Pacific region and Australasia (Boomer *et al.* 2012).

The long-standing taxonomic difficulties when describing species of *Mustelus* resonate in the field when scientists and fishers identify recognised species of *Mustelus* based on traditional morphological keys (Gardner and Ward 2002; Da Silva and Bürgener 2007; Akhilesh *et al.* 2014; Giresi *et al.* 2015; Marino *et al.* 2014). To this end, a number of smoothhound species are commonly misidentified and reported under the generic name smoothhounds and/or smoothhound sharks, gummy sharks, tollo or houndsharks. In the Mediterranean and Black Sea, *M. asterias*, *M. mustelus* and *M. punctulatus* (Farrell *et al.* 2009; Marino *et al.* 2014) are grouped, and similarly in Australia with *M. antarcticus*, *M. ravidus* and *M. stevensi* (Gardner and Ward 2002;

Boomer *et al.* 2012). Generic reporting of smoothhounds also occurs in the Northern Gulf of California (*M. albipinnis*, *M. californicus*, *M. henlei* and *M. lunulatus*; Pérez-Jiménez *et al.* 2013), Peru (*M. mento* and *M. whitneyi*; Romero *et al.* 2007) and in South Africa (*M. mosis*, *M. mustelus* and *M. palumbes*; Da Silva and Bürgener 2007; Da Silva *et al.* 2015). At present, efforts are underway to integrate taxonomic approaches (morphological and molecular) in an attempt to resolve the current taxonomic difficulties within *Mustelus*.

Gardner & Ward (2002) conducted the first integrative taxonomy study in *Mustelus* on species distributed across Australia and New Zealand. In that study, allozymes, mitochondrial RFLPs and precaudal vertebrae counts (Heemstra 1973) were used to distinguish species of *Mustelus*. Gardner & Ward (2002) found genetic support for three species of *Mustelus* in Australia (*M. antarcticus*, *M. stevensi* and *M. ravidus*) and one in New Zealand (*M. lenticulatus*). They also noted that genetic divergence among the whitespotted aplacental species (*M. antarcticus*, *M. lenticulatus* and *M. stevensi*) was low which was subsequently confirmed by Boomer *et al.* (2012). A fourth species of *Mustelus*, *M. walker*, in Australian waters has since been described (White and Last 2006, 2008). Likewise, Giresi *et al.* (2012*a*) reported a revised diagnostic key for identifying recognised species of *Mustelus* distributed in the western Atlantic Ocean, northern Gulf of Mexico. The later study used a molecular species identification approach based on the mitochondrial NADH-2 gene sequences and nuclear microsatellites as a guide first, and then identified easily ascertainable morphological characters that were able to discriminate between the genetically distinct clades. This included the labial furrow, ampullae of Lorenzini arrangement, lower lobe of the caudal fin, anterior nasal flaps and pectoral fin shape to distinguish among smoothhound sharks (*M. canis, M. norrisi* and *M.* sinusmexicanus) in the U.S. waters of the Gulf of Mexico. Subsequently, Giresi *et al.* (2015) reported a revised version of the dichotomous diagnostic key of Giresi *et al.* (2012*a*). Before that, Farrell *et al.* (2009) developed a molecular species identification assay for *M. asterias* and *M. mustelus*, occurring in the Northeast Atlantic Ocean, and a morphologically similar

triakid shark species *Galeorhinus galeus*. This gel-based assay was based on a multiplex polymerase chain reaction (PCR) consisting of a universal forward primer and three species-specific reverse primers for the NADH-2 region designed to amplify a fragment of different length for each species. Marino *et al.* (2014) adapted a similar approach while using the COI region, the internal transcribed spacer 2 (ITS2) region and microsatellites to identify the sympatric smoothhounds occurring in Adriatic basin of the Mediterranean Sea, *M. mustelus* and *M. punctulatus*. When the former methods of Marino *et al.* (2014) were applied to a larger sample set, only the microsatellites were able to provide conclusive results while the COI and ITS2 assays were not able to assign a fraction of the individuals, 6.6% and 14.4% respectively (Marino *et al.* 2017). The authors attributed the failure of the COI and ITS2 assays in specimen identification to the possible occurrence of heteroplasmy and introgression due to past hybridisation events. Also, Di Francesco (2011) and Marino *et al.* (2015) both used the same assays to identify *M. mustelus* × *M. punctulatus* hybrids, however, considering the study by Marino *et al.* (2017) the existence of *M. mustelus* × *M. punctulatus* hybrids remains questionable. Marino *et al.* (2017) also reported on morphological traits for practical specimen identification in the field including the shape of the dermal denticles, the distance of the nostrils and the shape of the mouth. For southern African sympatric smoothhound sharks, *M. mustelus* and *M. palumbes*, Maduna *et al.* (2017) in Chapter 6 only report on the molecular species identification based on distinctive allele frequencies at multiple microsatellite loci. All the above studies have guided the development of practical field identification keys for co-occurring smoothhound sharks and will further allow for the identification of cryptic biodiversity. This in turn could help to ensure that a critical level of biodiversity will be conserved within the genus *Mustelus* as it bridges the gap between intra- and interspecific variation (both morphological and molecular).

2.4.2. Mustelus *molecular ecology*

Smoothhounds are (epi)benthic coastal species that often have complex interactions with their near-shore habitats, related to foraging, mating, breeding and/or maturation (Espinoza *et al.* 2011; Francis *et al.* 2012; Da Silva *et al.* 2013). Such complex interactions can limit dispersal and result in geographically and/or genetically subdivided populations. Ideally, when a species exhibits little to no genetic structure in its local or global range (indicative of a single panmictic population or stock), it should be conserved as a single population (Dudgeon *et al.* 2012; Ovenden 2013). Phylogeographic and population genetic assessments of smoothhounds date back to the late 80's to early 2000's (MacDonald 1988; Gardner and Ward 1998; Chen *et al.* 2001) but have only recently begun to accumulate in literature (Pereyra *et al.* 2010; Boomer 2013; Chabot *et al.* 2015; Sandoval-Castillo and Beheregaray 2015; Maduna *et al.* 2016; Giresi unpubl data), contributing towards insights into the interplay of genetic drift and gene flow, and dispersal patterns in smoothhounds. Still, only eight out of 27 species of *Mustelus* have thus far been investigated to delineate the population genetic structure using neutral or near-neutral genetic markers (**Table 2.2**). The lack of genetic studies can partly be attributed to the limited available resources in some regions, but mostly to sampling difficulties where representative sample sizes for a population genetics approach may not be attainable.

Smoothhound species with relatively smaller distribution ranges and relatively high dispersal capacity (*e.g. M. antarcticus*, *M. lenticulatus* and *M. schmitti*) exhibit little or no detectable population genetic structure (Hendry 2004; Pereyra *et al.* 2010; Boomer 2013), whereas species with relatively wide distribution ranges exhibiting apparent site fidelity (*M. mustelus*) have higher levels of population genetic structure within and among bio-regions (Bitalo *et al.* 2015; Maduna *et al.* 2016). Conflicting results have also been presented between tagging and genetic data for *M. canis* and *M. henlei* for example. Based on mitochondrial DNA and microsatellite makers, Giresi

Table 2.2 Summary of key smoothhound population genetics studies. mtDNA refers to mitochondrial DNA sequences, RFLP denotes restriction fragment length polymorphism, SSR refers to simple sequence repeats (nuclear microsatellites), NADH-4 refers to mtDNA NADH dehydrogenase subunit 4 region, CR denotes mtDNA control region, while Cyt*b* refers to mtDNA cytochrome *b* region

Species	Study area	Data	Conclusions	Reference
Mustelus antarcticus	Southern and western Australia	Allozymes (mean of 32 loci)	Low genetic diversity, high gene flow between regions, female sex-biased dispersal	MacDonald (1988)
	Southern and eastern Australia	Allozymes (mean of 26.4 loci), mtDNA-RFLPs and vertebrae counts	High genetic diversity, two genetic stocks (southern and northern) although a third stock off Townsville might be present, no sex-biased dispersal	Gardner & Ward (1998)
	Southern and eastern Australia	NADH-4, 8 SSRs (species-specific, Boomer & Stow 2010)	Low genetic divergence, high population connectivity across regions, probable male sex-biased dispersal	Boomer (2013)
Mustelus canis	Atlantic and Gulf of Mexico	mtDNA, SSR	High genetic divergence and restricted gene flow between regions	Giresi (unpbl data)
Mustelus henlei	Gulf of California and the Pacific coast of Baja California	mtDNA-CR and 12 SSRs (cross-amplified, Boomer & Stow 2010)	Shallow genetic structure between regions was explained by spatial distance (but not by environmental factors), both female philopatry and male-biased dispersal impact on metapopulation structure.	Sandoval-Castillo & Beheregaray (2015)
	Gulf of California, Pacific coast of Baja California and Costa Rica	mtDNA-CR and 6 SSRs (species specific, Chabot 2012)	Genetic structure among three populations: northern (San Francisco), central (Santa Barbara, Santa Catalina, Punta Lobos, and San Felipe), and southern (Costa Rica).	Chabot *et al.* (2015)
Mustelus lenticulatus	New Zealand	Allozymes (mean of 17 loci), mtDNA-RFLPs	Low genetic diversity, no genetic differentiation detected although this does not necessarily mean that none exists	Hendry (2004)

Table 2.2 Continued.

Species	Study area	Data	Conclusions	Reference
	New Zealand	NADH-4, 8 SSRs (cross-amplified, Boomer & Stow 2010)	Low genetic divergence, high population connectivity across regions, no evidence for sex-biased dispersal.	Boomer (2013)
Mustelus manazo	Central Japan and northern Taiwan	Allozymes (20 loci)	Low genetic diversity, genetic structure between regions	Chen *et al.* (2001)
Mustelus Mustelus	South Africa	12 SSRs (cross-amplified, Byrne and Avise 2012; Chabot 2012; Giresi *et al.* 2012b)	Genetic structure, contemporary restricted gene flow across the Atlantic/Indian Ocean boundary	Bitalo *et al.* (2015)
	Southern Africa	NADH-4, 8 SSRs (cross-amplified, Byrne & Avise 2012; Chabot 2012; Giresi *et al.* 2012b)	Contrasting patters of genetic structure between SSRs and mtDNA implied at male philopatry, strong genetic structure between South-East Atlantic and South-West Indian Ocean samples was detected by microsatellites, while mtDNA suggested high population connectivity	Maduna *et al.* (2016)
	South Africa	11 SSRs (species specific, Maduna *et al.* 2017)	Validated inter-oceanic genetic structure and the role of the Atlantic/Indian boundary in restricting gene flow.	Maduna *et al.* (2017)
Mustelus palumbes	South Africa	11 SSRs (cross-amplified, Maduna *et al.* 2017)	Inter-oceanic genetic structure across the Atlantic/Indian boundary similar to *M. mustelus*.	Maduna *et al.* (2017)
Mustelus schmitti	Uruguayan Atlantic coast	mtDNA-Cyt*b*	Low levels of genetic diversity. Gene flow patterns indicated the existence of a single demographic unit in the Río de la Plata and its Maritime Front.	Pereyra *et al.* (2010)

(pers com) found distinct genetic populations in the western Atlantic and Gulf of Mexico for *M. canis* while Chabot *et al.* (2015) and Sandoval-Castillo & Beheregaray (2015) reported significant population genetic structure for *M. henlei* that is in contrast with previous tagging studies (Campos *et al.* 2009; Kohler *et al.* 2014). The inferred patterns of population structure in smoothhounds demonstrate that besides spatial scale, seascape features (hydrodynamic, thermal or ecophysiological boundaries), are driving genetic structure. Chabot *et al.* (2015) show that the Point Conception situated along the Pacific coast of the U.S. state of California, is a significant biogeographic barrier restricting gene flow between the northern and central populations of *M. henlei*. For southern African smoothhounds, Maduna *et al.* (2016, 2017) report that the Atlantic/Indian Ocean boundary, where the warm Agulhas Current collide with the cold upwelling Benguela Current, is responsible for the inter-oceanic population structure observed for *M. mustelus*. The role of seascape barriers in shaping genetic structure in sharks has been documented in a range of small-benthic to large-oceanic shark species. Examples include hydrodynamic barriers (open oceanic waters; Feldheim *et al.* 2001; Duncan *et al.* 2006; Schultz *et al.* 2008), thermal barriers (water temperature; Chabot and Allen 2009; Veríssimo *et al.* 2010) and biogeographic barriers (disconnection along continental coastlines; Duncan *et al.* 2006; Daly-Engel *et al.* 2012; Veríssimo *et al.* 2012; Vignaud *et al.* 2013). From these examples, it is clear that population genetic structure in smoothhounds is influenced by the interactions between evolutionary forces (gene flow and genetic drift), reproductive behavioural traits (philopatry, sex-specific dispersal) and/or seascape feature.

Tests for sex-biased dispersal (*i.e.*, where one sex is more dispersive than the other) using molecular data hinted at male-biased dispersal and female philopatry in *M. antarcticus* (Boomer 2013) and *M. henlei* (Sandoval-Castillo and Beheregaray 2015). These results contrast with earlier tag-based estimates of dispersal for these smoothhounds and/or closely related species that suggest that females are usually the dispersive sex, such as reported in *M. antarcticus* (Walker 1983; MaCDonald 1988), *M. lenticulatus* (Francis 1988), *M. schmitti* (Oddone *et al.* 2007), possibly also in *M. canis*

(Kohler *et al.* 2014), and *M. asterias* (Brevé *et al.* 2016). Contrasting maternally and bi-parentally inherited genetic markers in *M. mustelus* indicates high female-mediated gene flow (*i.e.*, dispersive females) and restricted male-mediated gene flow, which could be a manifestation of low levels of male straying (Maduna *et al.* 2016). Anecdotal reports of movement patterns in *M. mustelus*, however, did not detect sex-biased dispersal (Mann and Bullen 2009; Da Silva *et al.* 2013), although ongoing tagging and tracking studies suggest that females may have a higher dispersal potential than males (Da Silva C, perscomm). Yet, for *M. lenticulatus* there was no evidence for sex-biased dispersal based on molecular data (Boomer 2013). Generally, tagging studies only measure animal dispersal and do not detect whether migration has resulted in reproduction in the recipient population *i.e.*, interbreeding or effective dispersal (Ovenden 2013). Likewise, molecular genetic analysis often fails to detect dispersal depending on the effective population size versus the statistical approach employed (Palsbøll *et al.* 2007) or when there is a large stochastic variability in reproductive success (Hedgecock 1994; Frisk *et al.* 2014). Theoretically, the time for genetic drift to yield detectable population- and/or sex-specific genetic differentiation is typically on very different time scales relative to dispersal, rendering the translation of traditional population genetic approaches (gene flow) into the ecological equivalent measures (dispersal) complicated (Palsbøll *et al.* 2007). Furthermore, behavioural patterns of smoothhounds may differ among types of coastal habitats and environmental properties (Oddone *et al.* 2007; Campos *et al.* 2009; Espinoza *et al.* 2011; Da Silva *et al.* 2013). Determining whether a shark species explicitly exhibits sex-specific philopatry consequently requires efforts of continuous monitoring over several years (Hueter *et al.* 2005; Tillett *et al.* 2012; Feldheim *et al.* 2012). The value of using an integrated molecular ecology approach (genetic, tagging and tracking data) to better understand the movement patterns in smoothhounds is therefore apparent and recommended.

Another critical parameter to consider when evaluating the evolutionary consequences of the interplay between genetic drift and gene flow is the estimates of effective population size (*Ne*, Wright 1931, 1938, 1990; Schwartz *et al.* 1999). Unlike

census population size, *Ne* captures the effects of both demographic (fecundity, survival, mating system) and genetic (genetic drift, inbreeding depression, population bottlenecks) processes on natural populations, making it an important parameter for contemporary wildlife monitoring and management (Franklin 1980; Lande and Barrowclough 1987; Frankham 1995; Kamath *et al.* 2015). Obtaining reliable estimates of *Ne* could be challenging given that demographic data (*e.g.*, variables of the mating system, sex ratio and reproductive success) required for such calculations are often difficult to collect (Sollmann *et al.* 2012; Dudgeon and Ovenden 2015). Such estimates are also compounded by the dispersal regime (*e.g.*, sex-specific dispersal) and breeding tactic (*e.g.*, single- or multiple paternity) of a species (Byrne and Avise 2012; Lotterhos 2011). Remarkably, the exact effect of multiple paternity on *Ne* remains unresolved and highly debated in scientific literature (Murray 1964; Sugg and Chesser 1994; Karl 2008; Pearse and Anderson 2009; Lotterhos 2011). Murray (1964) originally asserted that multiple paternity (a single brood of offspring sired by multiple males) can maintain or increase *Ne*, a theory later supported by the theoretical framework of Sugg & Chesser (1994). Since then, Murray (1964) and/or Sugg & Chesser 1994 are often cited in support of the apparent positive effect of multiple paternity on genetic diversity and *Ne* in numerous publications without explicitly testing for this association (Robbins *et al.* 1987; Moran and Garcia-Vazquez 1998; Martinez *et al.* 2000; Garcia-Vazquez *et al.* 2001; Saville *et al.* 2002; Ferguson *et al.* 2013; Pirog *et al.* 2015; Bo *et al.* 2016; Gayet *et al.* 2016). Lotterhos (2011) demonstrates that the effects of multiple paternity on *Ne* will depend on the type of breeding systems that are compared (monogamous, single paternity or strictly monandrous).

Several recent studies in smoothhounds reported the presence of multiple paternity with variation in frequency between species and even populations (**Table 2.3**). Estimates of *Ne* remain largely unknown for smoothhounds (*sensu* Boomer 2013; Maduna 2014) compared to other well-studied species such as the bull shark *Carcharhinus leucas* (Tillett *et al.* 2012), white shark *Carcharodon carcharias* (Blower *et al.* 2012) and zebra shark *Stegostoma fasciatum* (Dudgeon and Ovenden 2015). It is

imperative that future studies also assess multiple paternity at the population-level and account in the least for the presence (and frequency) of multiple paternity following the method of Lotterhos (2011). Well-established DNA-based analytical methods (*e.g.*, linkage disequilibrium method) and software packages [namely LDNE (Waples 2006; Waples and Do 2008), NEOGEN (Blower in prep.) and NEESTIMATOR (Do *et al.* 2014; Jones *et al.* 2016)] for genetic estimation of *Ne* have been developed and could serve as a baseline when accounting for multiple paternity.

Table 2.3 Studies of multiple paternity (MP) in six species of smoothhound sharks. All assessments were made with microsatellites. N_L refers to the number of analysed litters while % MP indicates the percentage of litters presenting multiple paternity.

Species	Study area	N_L	% MP	Reference
Mustelus antarcticus	Western Australia, South Australia, Victoria, and New South Wales AUS	29	31	Boomer *et al.* (2013)
Mustelus asterias	Northeast Atlantic Ocean IRE	12	58	Farrell *et al.* (2014)
Mustelus henlei	Las Barrancas MX	14	93	Byrne & Avise (2012)
	Southern Pacific Ocean - Santa Catalina Island USA	18	22	Chabot & Haggin 2014
Mustelus lenticulatus	Manukau - northwest New Zealand	19	42	Boomer *et al.* (2013)
Mustelus mustelus	Mediterranean Sea - southern Tyrrhenian- and northern Adriatic regions IT	19	47	Marino *et al.* (2015)
Mustelus mustelus	Southwest Indian Ocean - Kwa-Zulu Natal RSA	6	67	Rossouw *et al.* (2016)
	Southwest Indian Ocean – Mossel Bay RSA	1	0	Chapter 7
Mustelus punctulatus	Mediterranean Sea - northern Adriatic region IT	13	54	Marino *et al.* (2015)

2.4.3. *Multiple paternity in* Mustelus

A series of field observations suggest that group reproductive behaviour and polyandrous copulations by females in a single mating event may be common in some sharks (Carrier *et al.* 1994; Yano *et al.* 1999; Pratt and Carrier 2001; Chapman *et al.* 2003). Female reproductive success is readily estimated in terms of the numbers of pups in a litter, but estimating the male's contribution is almost impossible. Male breeding success can, however, be estimated through paternity analysis. The results of several microsatellite analyses indicate that multiple paternity is a norm in smoothhound reproduction (**Table 2.3**). Multiple paternity in smoothhounds was first demonstrated in *Mustelus henlei* by Byrne & Avise (2012), with litters demonstrating the greatest incidence of multiple paternity – 93% of litters and an average number of sires = 2.3 – for any shark species (**Table 2.3**). Subsequently, Chabot & Haggin (2014) analysed 18 litters of *M. henlei* from Santa Catalina Island to determine whether this frequency is consistent elsewhere in the species' range. In the previous study, the authors detected multiple paternity in 22% of the study litters.

Overall, the pattern of multiple paternity observed between and within species provide several lessons about the reproductive behaviour in smoothhounds. First, reproductive mode (placental *vs.* aplacental viviparity) does not necessarily preordain the frequency of multiple paternity. Rossouw *et al.* (2016) argued that the differences in frequency of multiple paternity previously observed by Boomer *et al.* (2013) between placental and aplacental species of *Mustelus* is not a function of reproductive mode alone since *M. asterias* (aplacental species) had a similar incidence of multiple paternity to that of *M. mustelus* (placental species) (**Table 2.3**). Second, increased fecundity is not necessarily a driver of multiple paternity (Boomer *et al.* 2013). This suggests a lack of support for direct or indirect benefits of multiple paternity in smoothhounds and broader elasmobranchs (Feldheim *et al.* 2004; Portnoy *et al.* 2007; Dibattista *et al.* 2008; Daly-Engel *et al.* 2010; Verissimo *et al.* 2011). Third, there is no strong evidence to suggest that high mate encounter rates generated by male-biased breeding aggregations necessarily result in higher frequencies of multiple paternity

(Boomer *et al.* 2013). Fourth, geographic variation in the frequency of multiple paternity exists as observed in *M. henlei* (Byrne and Avise 2012; Chabot and Haggin 2014), *M. antarcticus* (Boomer *et al.* 2013) and *M. mustelus* (Marino *et al.* 2015; Rossouw *et al.* 2016). Finally, evidence of reproductive skew within polyandrous litters hints at postcopulatory mechanisms (*e.g.* long-term sperm storage). Indeed, several studies provide evidence for sperm storage in species of *Mustelus* including *M. antarcticus* (Storrie *et al.* 2008), *M. asterias* (Farrell *et al.* 2014), *M. canis* (Conrath and Musick 2002), *M. mosis* (Moore *et al.* 2016) and *M. mustelus* (Chapter 7). Long-term sperm storage allows females to exert cryptic choice (*i.e.*, controlled storage and release of sperm) which can influence male reproductive success (Thornhill 1983; Pearse and Avise 2001). Taken all of this into account, multiple paternity in smoothhounds is clearly not only guided by male density nor proclivity, or even the theoretical goals of female fitness and fecundity.

2.4.4. When conservation genetics enters the genomics era

There are already numerous articles that detail or critique specific aspects of the transition from conservation genetics to conservation genomics (Avise 2010; Helyar *et al.* 2010; Narum *et al.* 2013; Harrisson *et al.* 2014; Mcmahon *et al.* 2014; Willette *et al.* 2014; Shafer *et al.* 2015). The recent advancements in next-generation sequencing (NGS) technologies (so-called high-throughput, massively parallel sequencing), have made it feasible to identify large numbers (thousands to tens of thousands) of markers genome-wide at relatively low cost (Narum *et al.* 2013; Willette *et al.* 2014). A range of approaches enabling high-throughput genotyping-by-sequencing of single nucleotide polymorphisms (SNPs; Jiang *et al.* 2016) and microsatellite markers (Vartia *et al.* 2016; Farrell *et al.* 2016) are available and remain to be explored in conservation genomics studies. Conservation genomics depends on the simultaneous genotyping of a large number (thousands) of variable loci (SNPs and/or microsatellites) covering the entire genome rather than using 6–20 markers, as is routine in conservation genetics (Avise 2010; Shafer *et al.* 2015). Conservation genomics holds promise for a better

understanding of the interaction between various evolutionary processes, including mutation, random genetic drift, gene flow, and selection (Luikart *et al.* 2003; Coleman *et al.* 2010; Ogden *et al.* 2013). This genome-wide analysis provides a methodological approach for disentangling demographic (using neutral loci) from selection events (using putative adaptive or outlier loci). Demographic processes, such as bottlenecks, population expansions, degree of gene flow and random drift, are expected to affect genetic variation throughout the genome in a similar manner; whilst variation caused by mutation, selection or recombination are expected to deviate from genomic-background variation (Luikart *et al.* 2003). Incorporating genomic data into conservation can provide empirical assessment of the effect of smaller effective population sizes on functionally important genetic variation (Allendorf *et al.* 2010; Hazzouri *et al.* 2013). However, transitioning from conservation genetics to genomics is characterised by unresolved conceptual debates as to where genetics and genomics sit in relation to their application in the conservation of species. Particularly, why generate so much data when simple Sanger sequencing methods and downstream analyses may provide the information needed for conservation? (Helyar *et al.* 2010; Harrisson *et al.* 2014; Mcmahon *et al.* 2014; Shafer *et al.* 2015). In fact, to develop more rigorous conceptual, computational and theoretical frameworks that assist with the management of large datasets is proving to be difficult, hence, many genomics approaches end up with population genetics estimates *e.g.*, the fixation index F_{ST} (Hohenlohe *et al.* 2010; Rhode *et al.* 2013; Boehm *et al.* 2015; Portnoy *et al.* 2015). Nevertheless, population genomics has recently been applied in sharks to decipher demographic and adaptive process (Portnoy *et al.* 2015; Dimens 2016; Maisano Delser *et al.* 2016; Pazmiño *et al.* 2017), and resolve molecular taxonomy issues (Corrigan *et al.* 2017). Portnoy *et al.* (2015) studied a shark with a complex breeding tactic and dispersal system, the bonnethead shark (*Sphyrna tiburo*). In the former study, the authors used a population genomics approach together with sequences of the mitochondrial control region in bonnethead shark, a species thought to exhibit female philopatry. The study found that in species with sex-biased dispersal, reproductive

philopatry (return of adults to specific nursery sites to either mate or give birth) can facilitate sorting of locally adaptive variation, with the dispersing sex facilitating movement of potentially adaptive variation among locations and environments. The study of Corrigan *et al.* (2017) used 2152 nuclear SNPs along with the NADH2 marker to investigate genetic admixture between two superficially morphologically cryptic sharks, Galapagos shark (*Carcharhinus galapagensis*) and dusky shark (*Carcharhinus obscurus*). The NADH-2 marker failed to differentiate between these species while the SNP loci were consistent with the existing alpha taxonomy. In future, a similar approach may prove useful for understanding demographic and evolutionary processes in smoothhounds but for now it is critical to assess a larger number of species on a basic population genetics level.

2.5. Conclusions

In conclusion, the efforts to date are commendable and foundational; however, it is evident that there is still a considerable amount of work to be done in smoothhound conservation genetics. The systematics of *Mustelus* with the aim of resolving misidentification issues is gaining momentum, and will have major implications for effective species- and/or multispecies-level conservation. The use of both genetic and morphological characteristics to elucidate taxonomy (integrative taxonomy) is the way forward for the shark genus *Mustelus* as it offers the best chance of recording and protecting biodiversity. There is strong evidence of the interplay between evolutionary forces, life history traits and seascape features in shaping the patterns of intraspecific genetic diversity in smoothhounds. The actual sex-specific dispersal strategies in smoothhounds remain unclear given the discordance between genetic and tagging data. At present, it appears that sex-biased dispersal and the processes leading to it may be a matter of spatial scale and life history (*e.g.* migratory *vs.* non-migratory). A new paradigm has emerged from the genetic mating system and inferred reproductive behaviour studies in smoothhounds: assessing the presence or

absence of multiple paternity. As it remains uncertain as to what areas of the genome are important for principal evolutionary processes like speciation and adaptation to environmental conditions, taking a genomics approach would at least allow for genomic regions that are selectively neutral to be separated from those that are most likely affected by selection. This could provide more accurate and unbiased estimates of effective population size, rates and direction of gene flow and allow for the identification of adaptive diversity, all of which is necessary to inform shark conservation.

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2.7. Appendix

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CHAPTER 3

Historical biogeography of the shark genus *Mustelus* with reference to species from southern Africa

Abstract

Members of *Mustelus* display a widespread distribution pattern across ocean basins with a high degree of sub-regional endemism. The patterns and processes that resulted in smoothhound biodiversity and present-day distributions have not been explored in detail. From a conservation biogeography viewpoint, it is necessary to understand the past and present geographic patterns of a species in order to develop short and long-term conservation strategies. This chapter presents a regional species-level molecular phylogeny of *Mustelus* based on one nuclear gene (KBTBD2) and three mitochondrial DNA genes (CR, 12S-16SrRNA and NADH-2). In addition, the biogeographic origin of the genus in southern Africa is examined using a statistical historical biogeographic approach. Taxon sampling included seven species of *Mustelus* distributed across the eastern Atlantic and Indo-Pacific Oceans. Two additional triakid species, endemic to southern Africa, were included namely *Scylliogaleus quecketti* and *Triakis megalopterus*. In line with the earliest record of *Mustelus* in the northern hemisphere, results of the present study strongly support a northern hemisphere origin of southern African *Mustelus* species, with these species considered remnants of the Mediterranean Sea. It is also evident that the radiation of *Mustelus* in this region in all likelihood resulted from long-distance dispersal and that the Southern African species of the genus arose from at least two separate colonisation events from the Northern Hemisphere.

3.1. Introduction

Currently, there are nine orders, 34 families, 105 genera and 517 species of shark found throughout the world's coastal seas, oceans, and some freshwater systems (Weigmann 2017). The richness in biodiversity is currently under threat due to anthropogenic pressures including overexploitation, habitat degradation as well as climate change (Friedrich *et al*. 2014; Dulvy *et al*. 2008, 2017). Sixteen percent of shark species are considered threatened with extinction by the IUCN Red List's Shark Specialist Group (Dulvy *et al*. 2014, 2017). Consequently, numerous management and conservation measures including shark fin trade bans, fishing regulations (quotas, trip limits and size restrictions) and marine protected areas (Lack and Sant 2011; Biery *et al*. 2012; Shiffman and Hammerschlag 2016*a*, *b*; Ward-Paige 2017) have been put in place. At the same time, there has been an increase in the use of molecular tools to provide information on population, phylogeographic and demographic processes, some key elements in defining management units (Moritz 1994; Palsbøll *et al*. 2007).

Understanding past and present geographic patterns of a species is necessary to develop and implement viable conservation strategies. When assigning management units or placing protected areas for species conservation, several important considerations need to be addressed, which include (i) whether a location under consideration is part of the historic geographic range of the species or not, (iii) how and when the species did arrive, and (iii) whether the species is a cosmopolitan, an endemic or an exotic (Falk 1990; Brown and Lomolino 1998; Flather *et al*. 1998). These considerations can be readily addressed using historical biogeographic approaches. Historical biogeography is the field devoted to inferring the past geographic distributions of extant species while studying the processes that shaped these distributions (Crisci *et al*. 2001, 2003). The historical complexity underpinning present-day distribution patterns requires the application of molecular phylogenetics. This enables the evolution of a species to be traced on an explicitly hypothesised

cladogram, or more recently, a time-calibrated phylogeny (Matzke 2013*a*, *b*; Arias *et al*. 2017). This also constitutes a discipline called phylogenetic biogeography that was developed in the late 60s by Hennig (1966) and Brundin (1966).

Phylogenetic biogeography has undergone quite a revolution concerning its foundations, basic concepts and methods (Brundin 1966; Hennig 1966; Hovenkamp 1997, 2001; Ronquist 1997; Ree *et al*. 2005; Maguire and Stigall 2008; Arias *et al*. 2017). Of most importance was the development of event based methods, which include the parsimony-based dispersal–vicariance analysis (DIVA; Ronquist 1997) and the likelihood-based Dispersal-Extinction-Cladogenesis model (DEC; Ree 2005; Ree and Smith 2008). Several novel methods have since emerged, which include the Bayesian-based BayArea (Landis *et al*. 2013) and Bayesian Binary Model (BBM; Yu *et al*. 2013), a likelihood version of DIVA (DIVALIKE) and BayArea (Matzke 2013*a*, *b*), models for founder-event speciation (Matzke 2013*a*; Matzke 2014) and the geographically explicit event model (GEM; Arias *et al*. 2017). Each method relies, directly or indirectly, on some model of geographic range evolution and therefore entail specific assumptions about biogeographic processes or range inheritance scenarios, which include dispersal (range expansion), extinction (range contraction), sympatry, vicariance, and founder-event jump dispersal (Ree *et al*. 2005; Matzke 2013*a*; Arias *et al*. 2017). Several of these methods and corresponding techniques have conflicting assumptions (*e.g*. parsimony *vs*. likelihood). This creates a major problem on determining the best methods to use in a particular scenario (Matzke 2013*a*). Matzke (2013*a*, 2014) proposed the use of a likelihood framework to enable standard statistical model selection procedures (*e.g*., likelihood ratio test), implemented in the R package BIOGEOBEARS, to be applied for choosing the best model given the data.

Species of the shark genus *Mustelus* display quite a peculiar distributional pattern with a high degree of sub-regional endemism (Heemstra 1973; Weigmann 2016). This distribution pattern with high levels of endemicity in *Mustelus* is postulated to have occurred due to the interaction of different oceanographic features with the sedentary migration strategy of these species, thereby causing sudden subsidence and restriction

of species to isolated geographic ranges (Heemstra 1973; Boomer *et al.* 2012; Boomer 2013). Previous investigations into the phylogenetic relationship of *Mustelus* species using four protein-coding gene sequences (cytochrome *b*, cyt*b*; NADH dehydrogenase subunit 2 and -4, NADH-2 and NADH-4; recombination-activating gene 1, RAG1) and control region, provide compelling evidence that the genus is paraphyletic but constitutes a single monophyletic group when *Scylliogaleus quecketti* and *Triakis megalopterus* are included (López *et al.* 2006; Boomer *et al.* 2012; Naylor *et al.* 2012). These studies further show that members of *Mustelus* could be divided into two clades representing the different modes of reproduction (placental *vs.* aplacental species) coupled to the presence or absence of white spots on the dorsal surface (López *et al.* 2006; Boomer *et al.* 2012; Naylor *et al.* 2012). Interestingly, *M. punctulatus* and to a limited extent *M. mustelus*, have sparse black spots or blotches on their dorsal surface instead of white spots, the evolution of which is yet to be investigated (Farrell *et al.* 2009; Da Silva and Bürgener 2007; Marino *et al.* 2014). The biogeographic origin of the genus *Mustelus* in most regions is largely unknown, however, molecular phylogenies have proven useful in mapping the geographic origins of widespread species within the central Indo-Pacific region and Australasia (Boomer *et al.* 2012). In the latter study, based on molecular data from mitochondrial protein coding genes (NADH-2 and NADH-4), the northern hemisphere origin of *Mustelus* species is proposed, where a radiation following dispersal events from the north occurred (Boomer *et al.* 2012).

In southern Africa, there are two commercially important sympatric species of *Mustelus*, the common smoothhound *Mustelus mustelus* and the whitespotted smoothhound *M. palumbes.* Both these species are currently experiencing fishing pressures to support a demand for shark fins and fillets (Da Silva and Bürgener 2007; Da Silva *et al.* 2015). The common smoothhound is listed as 'Near-Threatened' by the IUCN Red-List (Serena *et al.* 2009) whilst the whitespotted smoothhound is listed as 'Data-Deficient' (Smale 2006). The common smoothhound is a cosmopolitan species with a widespread distribution from the Mediterranean Sea and eastern Atlantic Ocean to South Africa (Ebert *et al.* 2013; Weigmann 2016). The whitespotted

smoothhound shark however is endemic to southern Africa where the distribution extents from Namibia through South Africa (Eastern Cape Province, KwaZulu-Natal, Northern Cape Province, Western Cape) to southern Mozambique (Ebert *et al.* 2013; Weigmann 2016). In KwaZulu-Natal, these species co-occur with an isolated population of the north-western and north-eastern Indian Ocean species of *Mustelus*, the Arabian smoothhound *M. mosis* (Ebert *et al.* 2013; Weigmann 2016).

The evolutionary history of these species of *Mustelus* in southern Africa is currently unknown. In this chapter, the focus is primarily on the species of *Mustelus* of eastern Atlantic and Indo-Pacific Oceans to address questions regarding the origin and number of colonization events that resulted in the present-day southern African *Mustelus* species and whether any of these colonization events led to a species radiation. Also, the origin of black spots in the genus is investigated.

3.2. Materials and methods

3.2.1. Taxon sampling and distribution

Samples were collected from at least eight individuals for each of the five recognised species of the genus *Mustelus* (*M. asterias*, *M. mustelus*, *M. mosis*, *M. palumbes* and *M. punctulatus*; Weigman 2016) in north-eastern Atlantic Ocean including the Mediterranean Sea, south-eastern Atlantic and south-western Indian Oceans (**Figure 3.1, Table 3.1)**. Attempts at obtaining samples of *M. mosis* within the south-western Indian Ocean (South Africa) were unsuccessful; however, representative samples could be obtained from the north-western Indian Ocean (Oman). Given the paraphyly of the genus *Mustelus*, two additional whitespotted smoothhounds from the Indo-Pacific Ocean were also included as in-group taxa (*M. antarcticus* and *M. lenticulatus*) for a better representation of the 'aplacental' clade. Since López *et al*. (2006) and Naylor *et al*. (2012) noted that the genus *Mustelus* only constitutes a monophyletic grouping when *Scylliogaleus quecketti* and *Triakis*

megalopterus are included (see Chapter 2), these species were also included as in-group taxa. *Iago omanensis* was selected as an outgroup taxon for phylogenetic analysis.

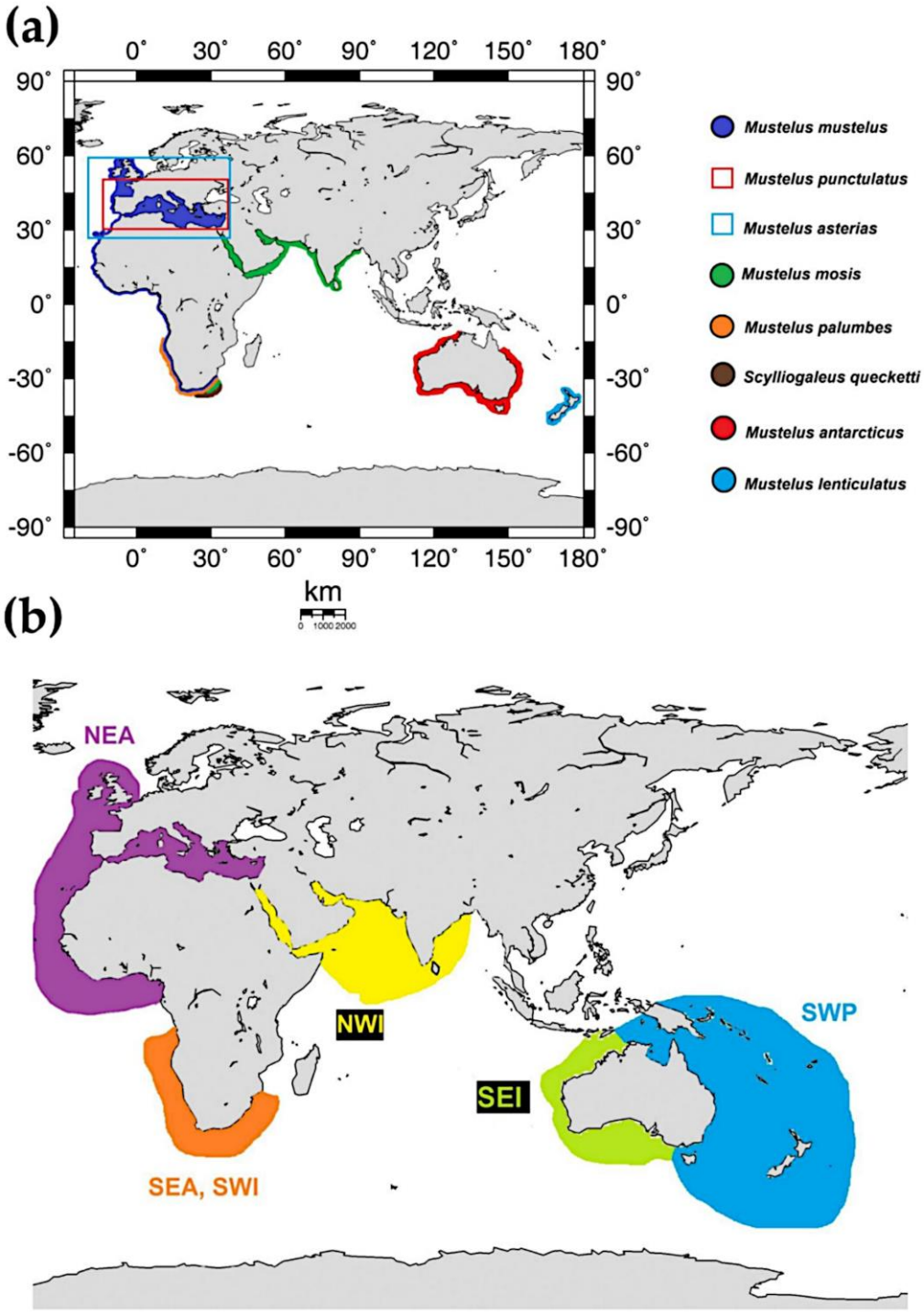

Figure 3.1 Distribution (a) and biogeographic areas (b) of study taxa according to Ebert *et al*. (2013) and Weigmann (2016), respectively (see Chapter 2, **Table 2.1**). SEI, south-eastern Indian Ocean; NEA, north-eastern Atlantic Ocean including the Mediterranean Sea; NWI, north-western Indian Ocean; SEA, south-eastern Atlantic Ocean; SWI, south-western Indian Ocean; SWP, south-western Pacific Ocean.

Table 3.1 Summary of the species and the number of individuals (*N*) per species per location included in the present study.

Species	Code	*N*	Locality	Collector
Mustelus antarcticus	Man	8	Western Australia	Jessica J. Boomer (JJB)
Mustelus asterias	Mas	8	United Kingdom, Wales	Edward D. Farrell (EDF)
Mustelus lenticulatus	Mle	8	New Zealand	JJB
Mustelus mosis	Mmo	8	Sultannate of Oman	Mikhail V. Chesalin (MVC)
Mustelus mustelus	Mmu	2	Mediterranean, Adriatic Sea	Ilaria A. M. Marino (LAMM)
		2	Mediterranean, Tyrrhenian Sea	LAMM
		4	Mediterranean, Balearic Islands	EDF
		1	Guinea-Bissau	Ana Verríssimo (AV), Chrysa Gubili (CG)
		1	Guinea-Conakry	AV, CG
		2	Cape Verde	AV, CG
		8	Angola	Michelle Soekoe
		1	Namibia	AV, CG
		2	South Africa, Langebaan Lagoon	Charlene da Silva
		2	South Africa, False Bay	MS
		2	South Africa, Mossel Bay	Gibbs Kuguru
		2	South Africa, Durban	KZN Sharks Board

Table 3.1 Continued.

Species	Code	*N*	Locality	Collector
Mustelus palumbes	Mpa	12	South Africa, West Coast	Grant van der Heever
		1	South Africa, Mossel Bay (East Coast)	Gibbs Kuguru
		3	South Africa, Struis Bay (East Coast)	Viking Fisheries
Mustelus punctulatus	Mpu	4	Algeria, Mediterranean Sea	EDF
		4	Croatia, Adriatic Sea	EDF
Scylliogaleus quecketti	Squ	8	South Africa, Durban	Bruce Mann
Triakis megalopterus	Tme	2	Angola	Michelle Soekoe
		2	Namibia	Michelle Soekoe
		3	South Africa, West Coast	Michelle Soekoe
		3	South Africa, East Coast	Michelle Soekoe
Iago omanensis	Iom	4	Sultannate of Oman	MVC

3.2.2. DNA extraction, quantification and visualization

Genomic DNA extraction was carried out with a modified cetyltrimethylammonium bromide (CTAB) method (Sambrook and Russell 2001). A small piece of fin clip or muscle tissue was mixed with 150 µL of the CTAB extraction solution [2% (w/v) CTAB, 0.02 M ethylenediaminetetraacetic acid (EDTA), 0.1M tris(hydroxymethyl)aminomethane (Tris), 1.4M sodium chloride, 0.2% (v/v) β-mercaptoethanol, adjusted to pH 6.5 with 4M hydrochloric acid, autoclaved] and 20 µL proteinase K solution (10 mg/mL). The mixture was incubated in a waterbath (model WNB 7, Memmert, Lasec, South Africa) at 56 °C for 4 hours and inverted every 30 minutes. Subsequent to incubation, an equal volume of chloroform: isoamyl alcohol (24:1) was added and the solution was mixed by inversion for 1 min. After centrifugation at 16 000 *g* (model 5415 D, Eppendorf, South Africa) for 5 min, 140 µL of the aqueous phase was transferred into a new 1.5 mL Eppendorf tube that already contained 140 µL chloroform/iso-amylalcohol (24:1, v/v). After mixing by inversion for 1 min, the solution was centrifuged at 16 000 *g* for 5 min. Approximately 120 µL of the aqueous phase was transferred into a new 1.5 mL Eppendorf tube, 2 volumes (240 µL) of ice-cold absolute ethanol was added and the tube was inverted several times prior to incubation at -20 °C overnight. Using a refrigerated centrifuge (model 5810, Eppendorf, South Africa), tubes were centrifuged at 4°C at 14 610 *g* for 20 min. The supernatant was decanted, and 200 µL of 70% (v/v) ethanol was added to the tube containing the DNA pellet. The tubes were inverted several times and incubated at room temperature for 10 min. After a further centrifugation step (4°C, 14 610 *g*, 10 min), the supernatant was carefully removed with a pipette and the tubes were left uncapped in the fumehood for 3 hours to allow for the pellets to dry. Deoxyribonucleic acid pellets were then resuspended in 50 µL double-distilled water under shaking at room temperature for 2 hours. The concentration and the quality of the DNA were determined by measuring its optical density at 260 nm (A_{260}) and 280 nm (A_{280}) with a NanoDrop ND 2000 spectrophotometer (Thermo Fisher Scientific;

wwwthermofisher.com). A small subset of samples was subjected to electrophoresis in 1× TAE buffer for 1 hour at 80 V. Ten microlitre of the isolated genomic DNA was loaded on 0.8% agarose gel stained with ethidium bromide to check DNA quality. The gels were photographed under a Gel Documentation system (Gel Doc XR+, Bio-Rad, South Africa).

3.2.3. Polymerase chain reaction and sequencing

Three mitochondrial genes (NADH dehydrogenase subunit 2, NADH-2; control region, CR; 12S-16S ribosomal DNA, 12S-16S) and two nuclear genes (kelch repeat and BTB domain containing 2, KBTBD2; internal transcribed spacer 2, ITS2) were amplified using previously reported universal primers (**Table 3.2**). Polymerase chain reaction (PCR) was carried out on a GeneAmp® PCR System 2700 in a 15 µL reaction volume that included 100 ng of template DNA, 1x PCR Buffer, 200 µM of each dNTP, 0.5 µM of each primer (except for KBTBD2, 0.3 µM), 2.5 mM $MgCl_2$ and 0.5 U of GoTaq® DNA polymerase. The specific PCR cycling conditions for CR, NADH-2, 12S-16S and ITS2 were according to Boomer *et al*. (2012), Iglésias *et al*. (2005) and Pank *et al*. (2001), respectively. For the nested PCR of the KBTBD2 gene, the optimum annealing temperatures and PCR cycling profiles were determined in this study. For the nested PCR, the PCR cycling conditions for this gene were as follows: (i) one cycle of initial denaturation at 95°C for 2 min, (ii) 35 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s, elongation at 72°C for 1 min, and (iii) a final elongation of one cycle at 72°C for 5 min. Amplification products were sized using agarose gel electrophoresis and bi-directionally sequenced and thereafter stored at 4°C. Cycle sequencing was conducted in a total volume of 10 µL using the standard Sanger sequencing chemistry (BigDye® terminator v3.1 cycle sequencing kit; Life Technologies) and 1 pmol of each primer following the manufacturer's instructions. Capillary electrophoresis was conducted at the Central Analytical Facility, Stellenbosch University.

Table 3.2 DNA markers used for molecular phylogenetic analyses.

DNA region	Primer name	Sequence	Forward/reverse	References
Mitochondrial				
NADH-2	ILEM	5'-AAGGAGCAGTTTGATAGAGT-3'	F	Naylor *et al*. (2005)
	Ilem-*Mustelus*	5'-AAGGACCACTTTGATAGAGT-3'	F	Naylor *et al*. (2005)
	Asn-*Mustelus*	5'-AACGCTTAGCTGTTAATTAA-3'	R	Naylor *et al*. (2005)
CR	MaCybF	5'-TAACTTGAATTGGRGGRCAAC-3'	F	Boomer *et al*. (2012)
	MaDLPR	5'-GCATTAATCAGATGYCAGRT-3'	R	Boomer *et al*. (2012)
12S-16S	Chon-Mito-S005	5'-AGGCAAGTCGTAACATGGTAAG-3'	F	Iglésias *et al*. (2005)
	Chon-Mito-R017	5'-ATCCAACATCGAGGTCGTAAACC-3'	R	Iglésias *et al*. (2005)
Nuclear				
KBTBD2	KBTBD2_F9	5'-TGTATTGCATCGTTGTCGRGAATA-3'	F (nested-PCR round 1)	Li *et al*. (2012)
	KBTBD2_R1522	5'-GCAATGCAACCATCTCTCCATC-3'	R (nested-PCR round 1)	Li *et al*. (2012)
	KBTBD2_F49	5'-AATGCAGAGAACTGYGTGCGAT-3'	F (nested-PCR round 2)	Li *et al*. (2012)
	KBTBD2_R1515	5'-AACCATCTCTCCATCCAAYTCAAACTC-3'	R (nested-PCR round 2)	Li *et al*. (2012)
ITS2	FISH5.8SF	5'-TTAGCGGTGGATCACTCGGCTCGT-3'	F	Pank *et al*. (2001)
	FISH28SR	5'-TCCTCCGCTTAGTAATATGCTTAAATTCAG-3'	R	Pank *et al*. (2001)

3.2.4. Sequence alignment and phylogenetic analyses

Alignment of forward and reverse sequences, base-calling, end-clipping and ambiguity checks were carried out in CODONCODEALIGNER v. 6.0.2 (CodonCode Corporation) using the ClustalW algorithm of Thompson *et al*. (1994) for alignment. For nuclear genes, heterozygote positions were coded according to the universal ambiguity code. Full sequence alignments for each gene were produced in BIOEDIT v. 7.2.5 (Hall 1999) using the contig assembly program of Huang (1992). Sequences were aligned using the online version of MAFFT v. 7.299 (Katoh and Standley 2013), with the FFT-NS-i algorithm for the protein coding genes and Q-INS-i for ribosomal fragments (Katoh and Toh 2008). The correct translation to amino acids was checked in MEGA v. 7.0.14 (Kumar *et al*. 2016). After alignment, introns and other positions of ambiguous alignment were removed in GBLOCKS v. 0.91b (Castresana 2000) using the following parameters: (i) minimum length of a block after gap cleaning was set to 10, (ii) no gap positions were allowed in the final alignment, (iii) all segments with contiguous non-conserved positions bigger than 8 were rejected and (iv) minimum number of sequences for a flank position was set to 85%. The aligned, individual gene data sets were concatenated using SEQUENCEMATRIX (Vaidya *et al*. 2011). Locus-specific nucleotide substitution models were chosen using jModelTest v. 2.1.2 (Guindon and Gascuel 2003; Darriba *et al*. 2012). For the concatenated dataset, PartitionFinder v. 2.1.1 (Lanfear *et al*. 2012) was used to select the best-fitting partitioning scheme and models of evolution using the Bayesian Information Criterion (BIC). This entailed the greedy algorithm and unlinked branch lengths for the different markers and/or their codon positions if a coding sequence was involved.

Maximum parsimony (MP) analyses were conducted using Fitch parsimony with equal weighting of all characters and of transitions/transversions as implemented in PAUP* v. 4.0*a* (Swofford 1991). Heuristic searches were replicated 100 times with random taxon-addition sequences, tree bisection-reconnection (TBR) branch swapping, and the options Multrees and Steepest Descent in effect. Support for

monophyletic groups was assessed by bootstrapping (1000 resamplings of the data) using the heuristic search strategy. Maximum likelihood (ML) phylogeny reconstruction was conducted with PHYML v. 3.0 (Guindon and Gascuel 2003). This entailed using the Nearest Neighbor Interchange (NNI) approach to search for tree topology and computing an approximate likelihood-ratio test (*aLRT*) for branch support instead of a non-parametric bootstrap test (Anisimova and Gascuel 2006). Bayesian phylogenetic inference was only performed for the concatenated dataset using MrBAYES v. 3.2.6 (Huelsenbeck *et al*. 2001; Ronquist 2004). A pair of independent searches for 1 million generations was run, with trees saved every 1000 generations and the first 250 sampled trees of each search discarded as burn-in, with the corresponding model of evolution each gene region (HKY+I+G for CR, HKY+G for NADH-2 and 12S-16S, HKY for KBTBD2). Finally, a 50% majority rule consensus tree was constructed. All phylogenetic reconstruction analyses except ML analysis were performed as implemented on Cyberinfrastructure for Phylogenetic Research (CIPRES) Science Gateway portal v. 3.3 (www.phylo.org) at the San Diego Supercomputer Center (Miller *et al*. 2010).

3.2.5. Ancestral area reconstruction

The historical biogeographic ranges of the southern African *Mustelus* species were reconstructed using BIOGEOBEARS (Matzke 2013*a*, *b*) in R (R Core Team, 2016). Species were assigned to one or more of the following biogeographic realms according to their current distributions (Weigmann 2016); eastern Indian Ocean, north-eastern Atlantic Ocean including the Mediterranean, north-western Indian Ocean, south-eastern Atlantic Ocean, south-western Indian Ocean and/or south-western Pacific Ocean (see **Figure 3.1**). The maximum range size was set to 5 as no extant species occurs in more than five of the biogeographic realms. The following models of geographic range evolution were compared in a likelihood framework. First, a Dispersal-Extinction Cladogenesis Model (DEC) as originally implemented in the software LAGRANGE (Ree and Smith 2008) was used. It has two free parameters

specifying the rate of 'dispersal' (*i.e.*, range expansion) and 'extinction' (*i.e.*, range contraction), while the cladogenesis model remains fixed. This means that the geographical range of the ancestral lineage is inherited with equal probability by the two daughter lineages through a variety of plausible cladogenenetic scenarios (*e.g.*, sympatry, parapatry, vicariance). Next, the DEC + j model (Matzke, 2013*b*; Matzke 2014), which adds a third free parameter to the Dispersal-Extinction Cladogenesis (DEC) framework, that of long-distance dispersal (parameter j – DEC + j model), was used. This effectively mimics the process of founder-event speciation as one daughter lineage can disperse to an area beyond the ancestral range. The classic DEC model is nested within the DEC + j. Dispersal Vicariance Analysis (DIVA) (Ronquist 1997) and DIVA with founder parameter (DIVA + j) (Matzke 2013*b*) were also implemented. Additionally, the Bayesian inference of historical biogeography for discrete areas (BAYAREA) (Landis *et al*. 2013), and BAYAREA with founder parameter (BAYAREAj) (Matzke 2013) was also tested. Model fit was assessed using the Akaike information criterion (AIC).

3.3. Results

Initial optimisation of the selected phylogenetic markers showed that all gene regions except the ITS2 could be amplified and sequenced successfully. From initial screenings of a subset of the data, the CR was the most variable (mean evolutionary distance 7.9%, 0.9% standard errod (SE)) and amplified consistently across the study taxa. A total of 107 samples/taxa representing seven species of *Mustelus* plus two triakid species and one outgroup species were sequenced for the CR and the nuclear gene KBTBD2 (mean evolutionary distance 1.3%, 0.8% SE). For the two mitochondrial DNA regions (NADH-2 and 12S-16S) however, only a subset of samples could successfully be sequenced. Concatenated data included 528 base pairs (bp) CR, 341 bp NADH-2, 370 bp 12S-16S and 1347 bp KBTBD2. Four out of seven samples designated

as *Mustelus mosis* (Mmo) were identified as *Iago omanensis* using the NADH-2 gene region, and these samples were used as outgroups.

The individual gene datasets resulted in somewhat different topologies largely due to differences in taxon density and coverage with the CR and the NADH-2 trees being most similar (**Figures 3.2 – 3.9**). Both the ML and MP trees for the CR dataset alone supported the separation of the genus *Mustelus* into two major clades (placental and aplacental clades; **Figures 3.2** and **3.3**). For the combined dataset, the 50% majority-rule consensus tree generated by MrBAYES (**Figure 3.10**) also supported this phylogeographic structure (separation into two major clades) of *Mustelus* and confirmed the monophyletic nature of expanded *Mustelus*.

As expected, *Mustelus* was only supported as a monophyletic clade when *S. quecketti* and *T. megalopterus* were included. The blackspotted smoothhound *M. punctulatus* was nested within the placental non-spotted clade and its basal placement in this clade suggests the secondary loss of black spots in the genus. Further supporting this hypothesis was the sister-group relationship of the genus *Mustelus* with the black spotted gully shark *T. megalopterus*. On a microevolutionary scale, evidence for phylogeographic structure for the common smoothhound *Mustelus mustelus* was uncovered (**Figure 3.2**). Interestingly, samples from Cape Verde were nested within the southern African clade, while the samples from Guinea-Bissau and Guinea-Conakry were in a subclade within the Mediterranean clade (**Figure 3.2** and **3.10**)

The best model describing range evolution in the expanded *Mustelus* was the DEC + j model (log likelihood: LnL = -4.06; parameter estimates: d = 1 E^{-12}, e = 1 E^{-12} and j = 3.0). Based on this model, the most recent common ancestor of the southern African *Mustelus* originated in the Northern hemisphere, with a distribution in the north-eastern Atlantic Ocean including the Mediterranean Sea as evident in **Figure 3.2**.

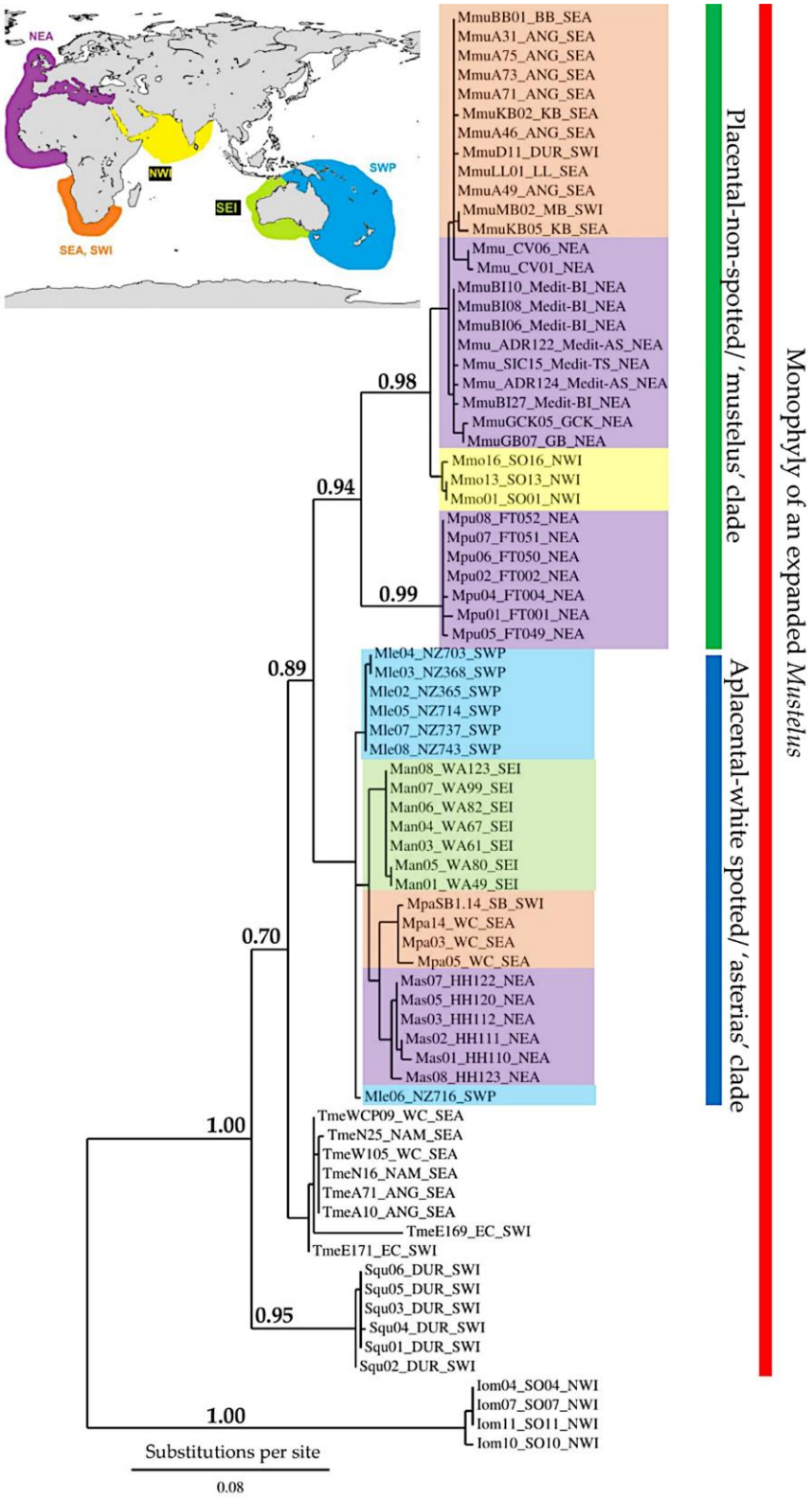

Figure 3.2 The best-scoring ML tree derived from the control region data set. Coloured bars represent the placental and aplacental clades and the monophyly of an expanded *Mustelus* (including *Scylliogaleus quecketti* and *Triakis megalopterus*), respectively. The numbers next to the nodes indicate ML bootstrap support >65%. As inset the biogeographic areas are shown. See Figure 3.1 and Table 3.1 for area codes and species codes.

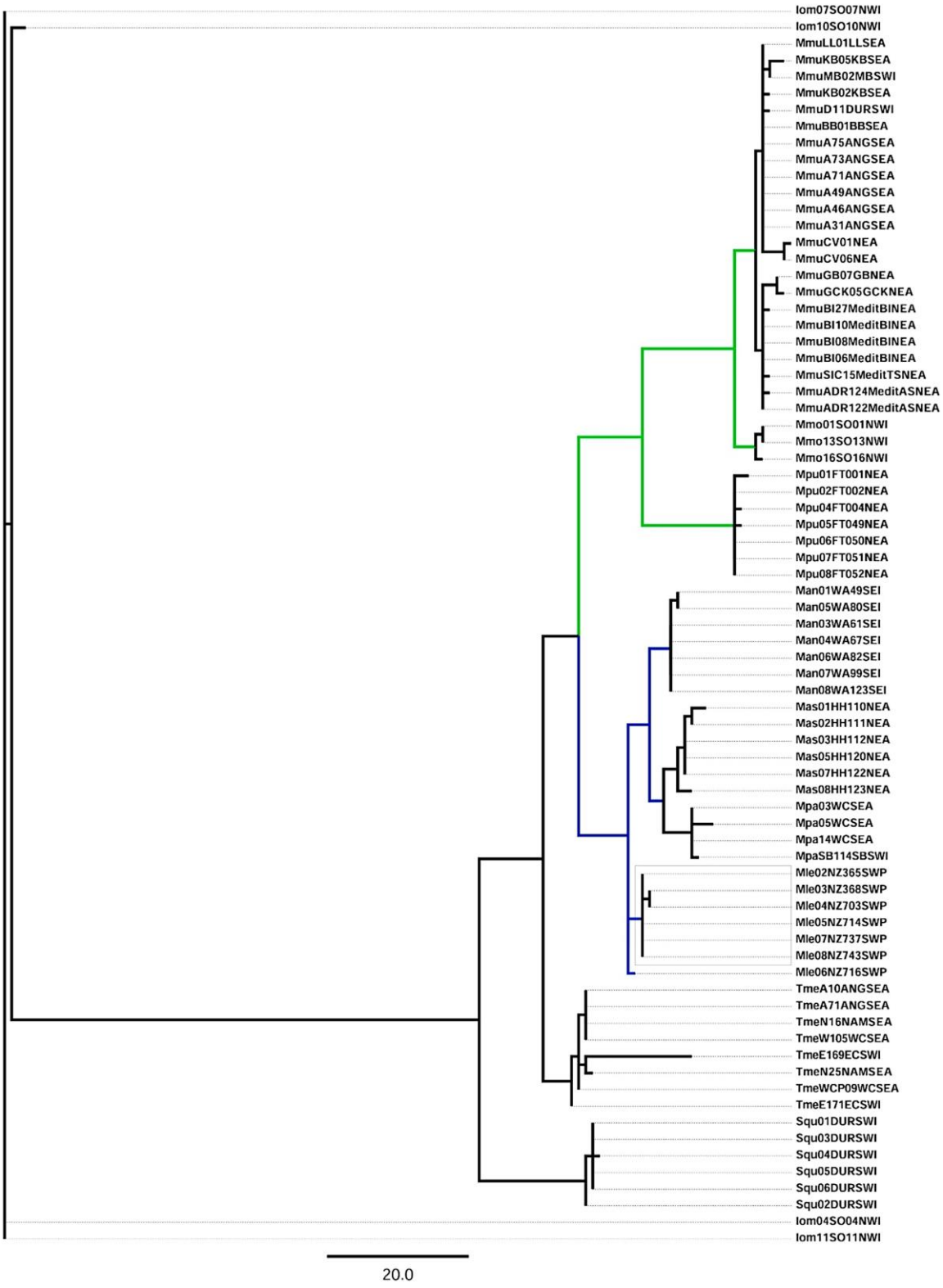

Figure 3.3 Most parsimonious tree for expanded *Mustelus* based on the control region data set. Green and blues branches represent the placental and aplacental clades, respectively. See **Table 3.1** for species codes.

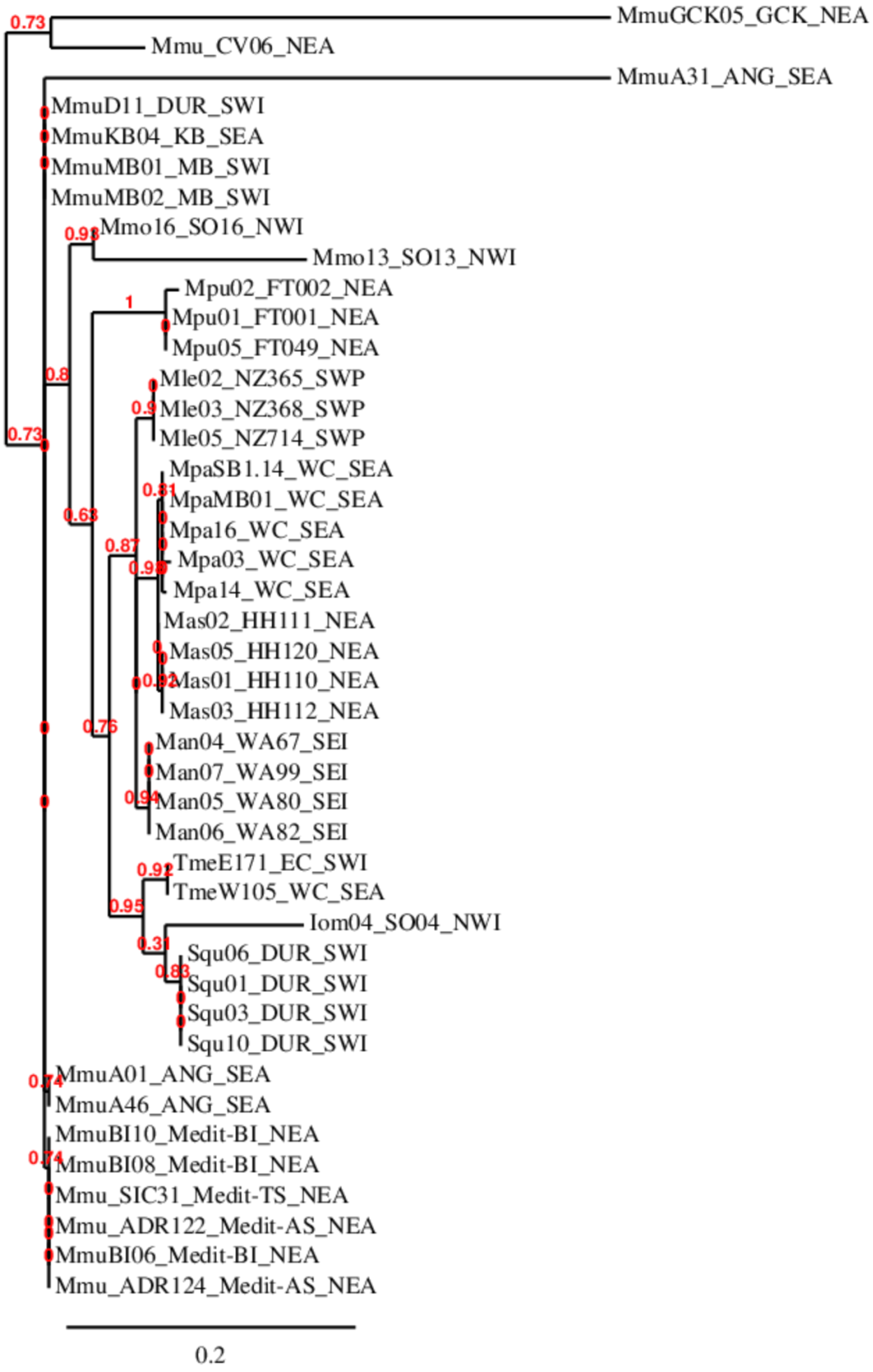

Figure 3.4 The best-scoring ML tree derived from the NADH-2 data set.

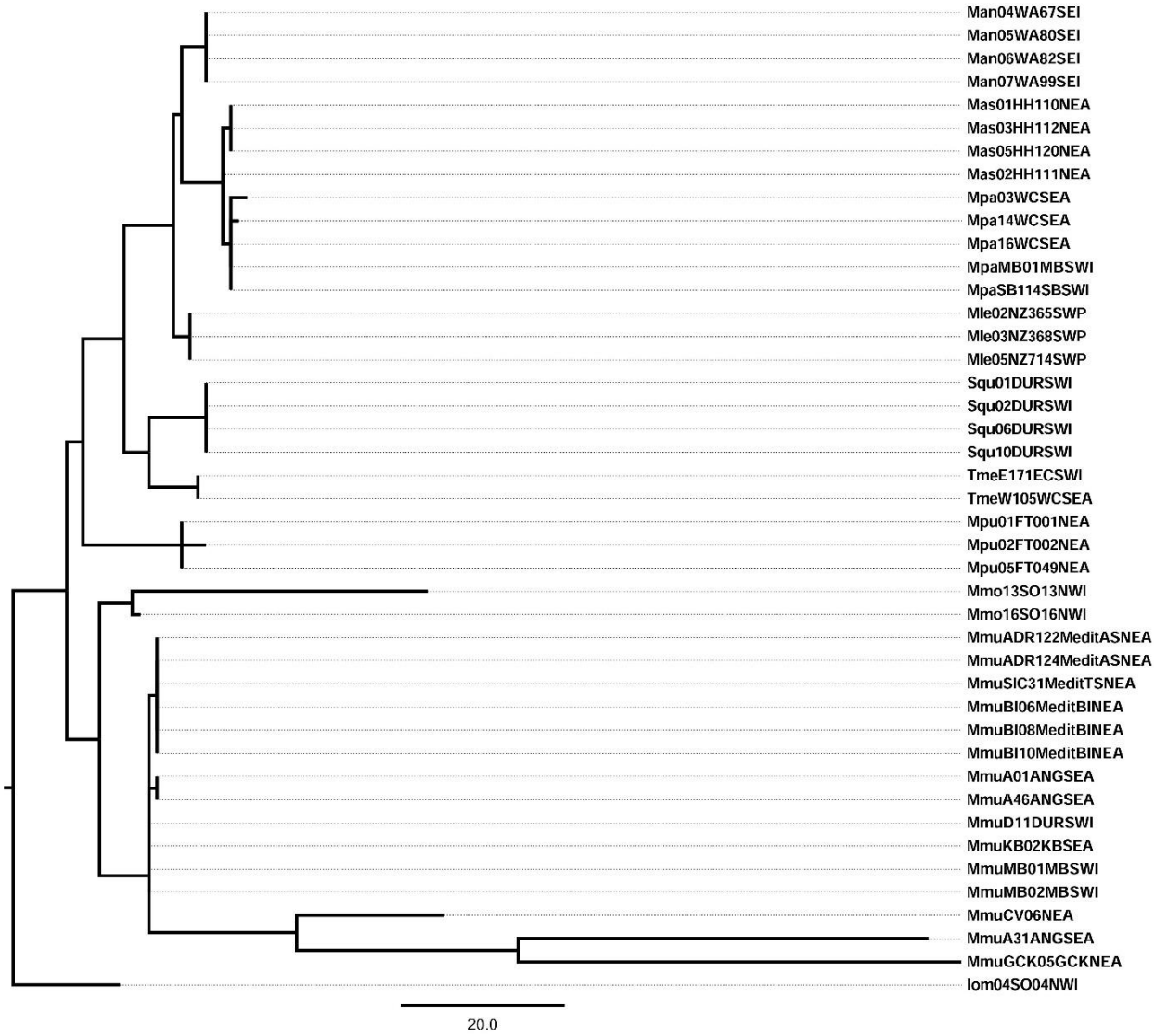

Figure 3.5 Most parsimonious tree for expanded *Mustelus* based on the NADH-2 data set. See **Table 3.1** for species codes.

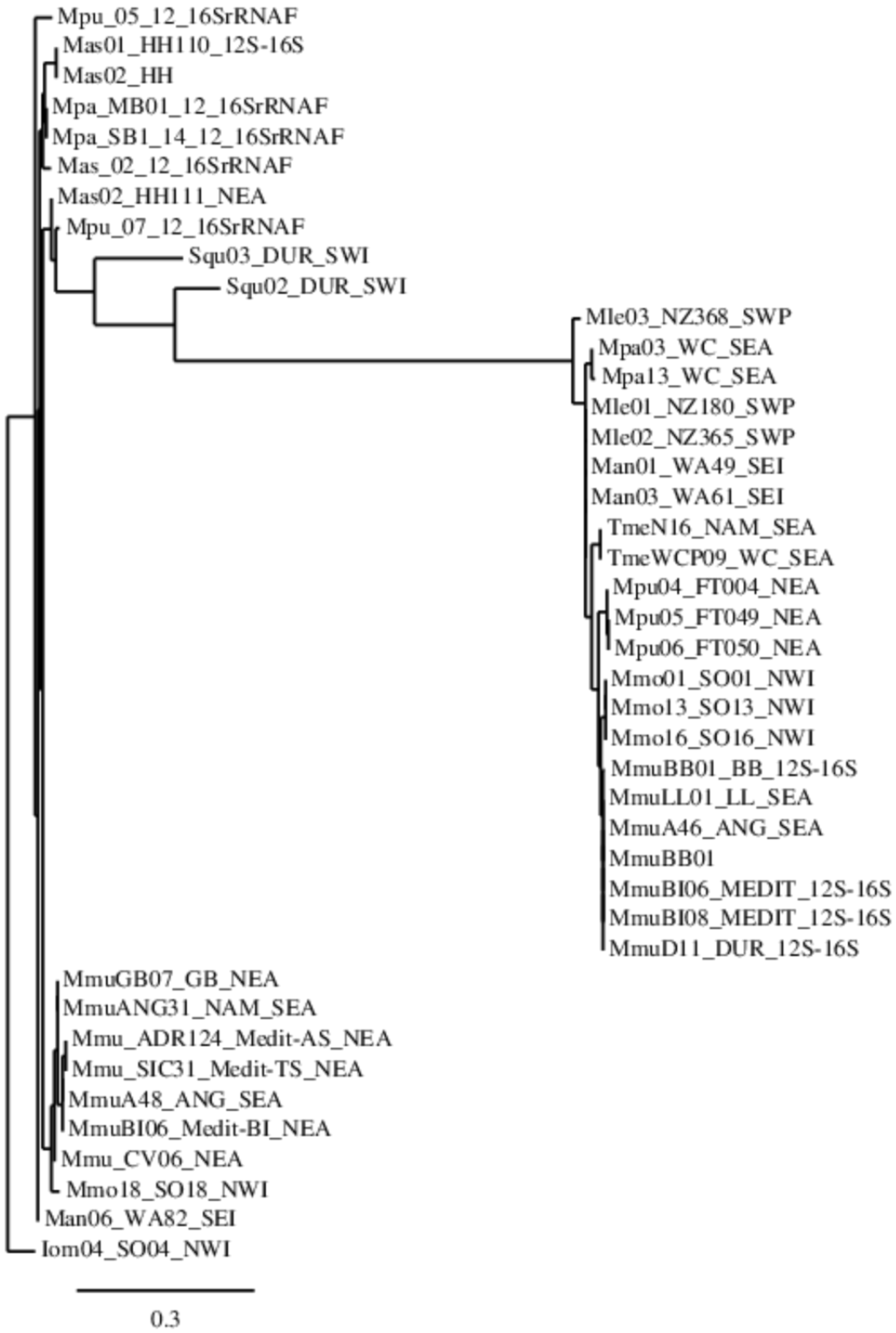

Figure 3.6 The best-scoring ML tree derived from the 12S-16S data set.

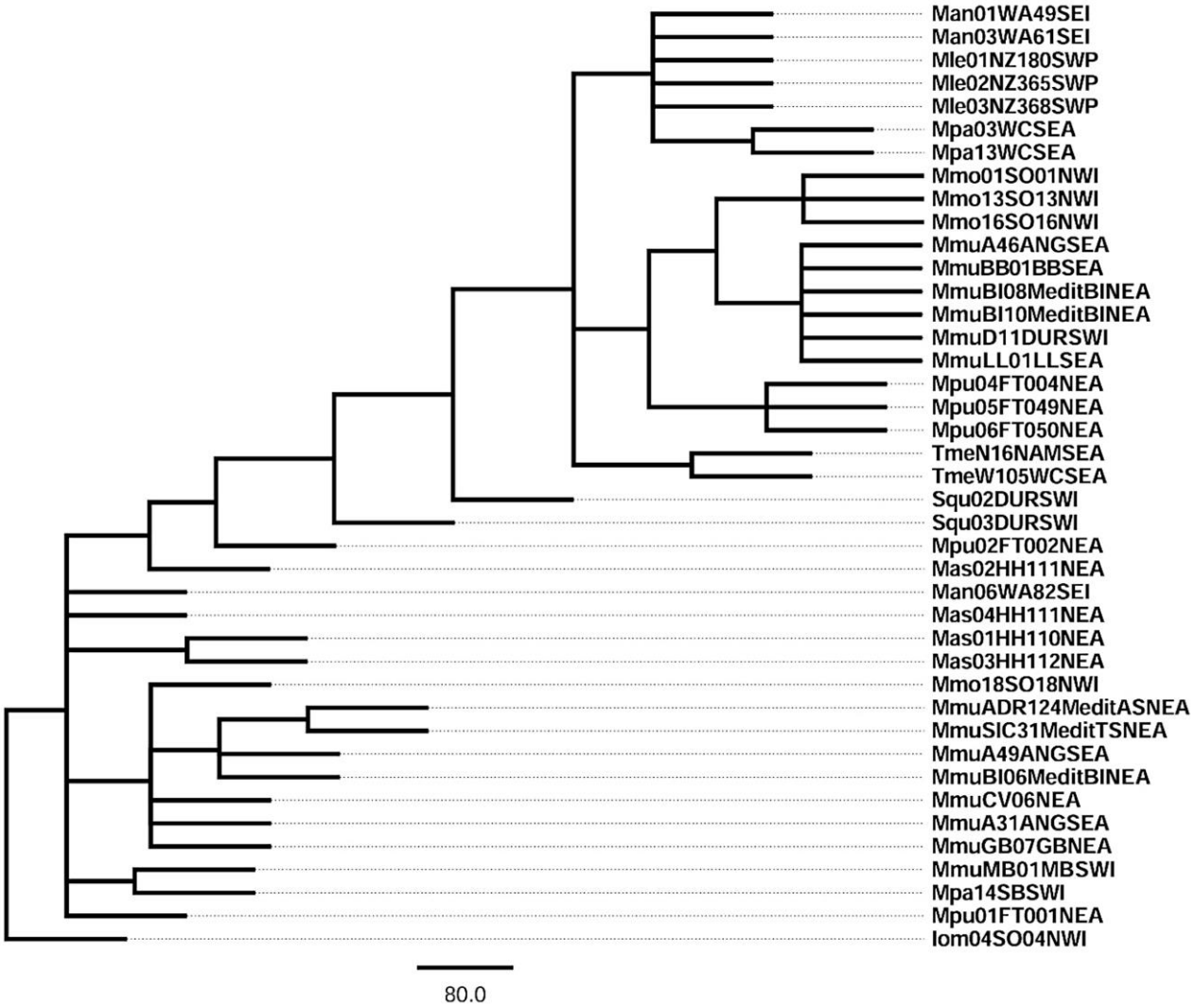

Figure 3.7 Most parsimonious tree for expanded *Mustelus* based on the 12S-16S data set. See **Table 3.1** for species codes.

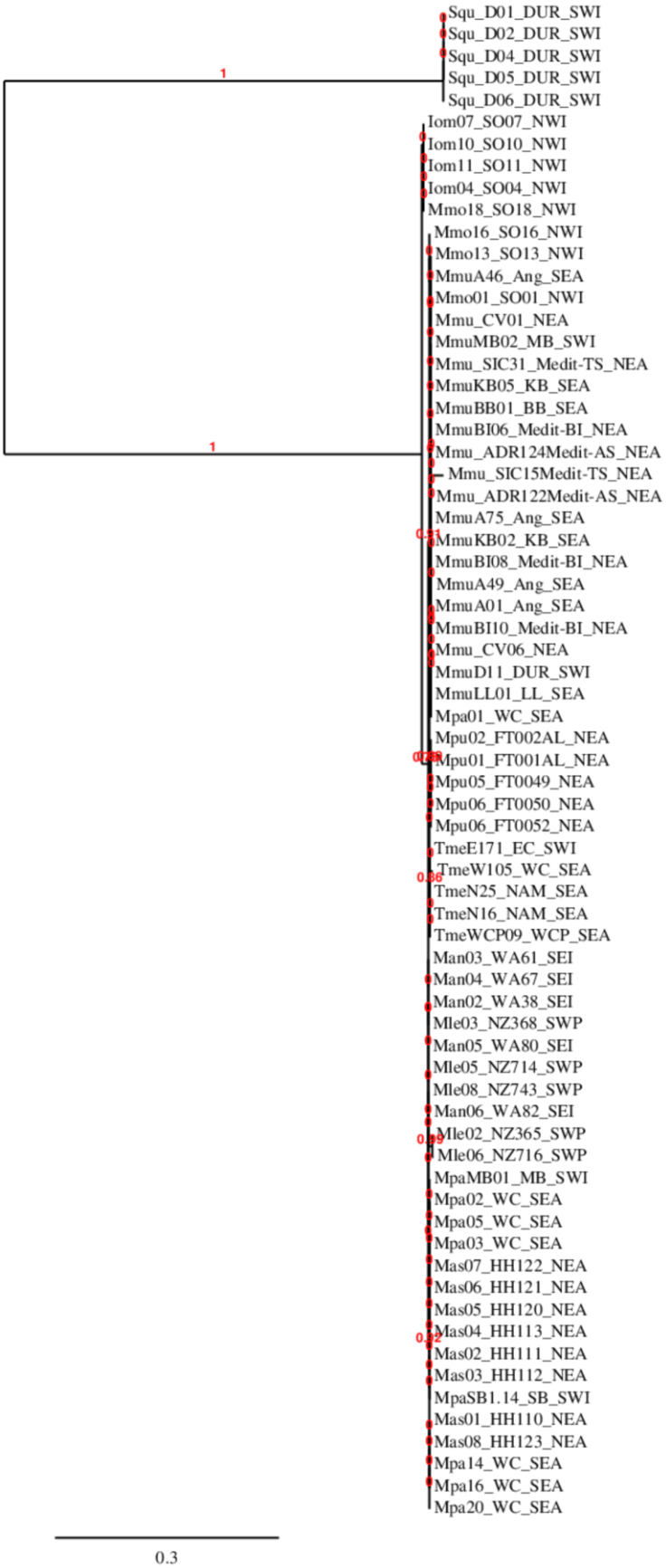

Figure 3.8 The best-scoring ML tree derived from the KBTBD2 data set.

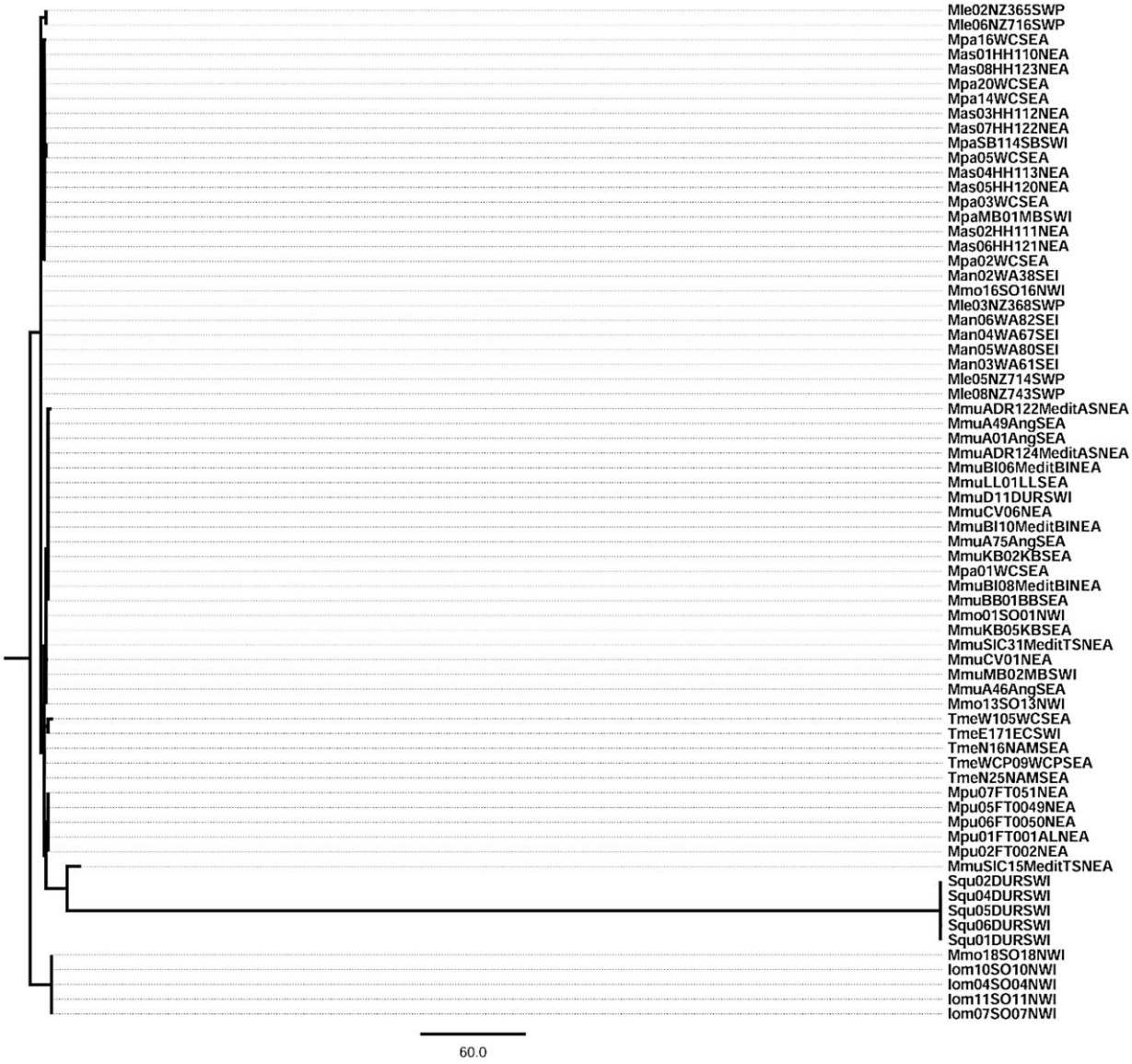

Figure 3.9 Most parsimonious tree for expanded *Mustelus* based on the KBTBD2 data set. See **Table 3.1** for species codes.

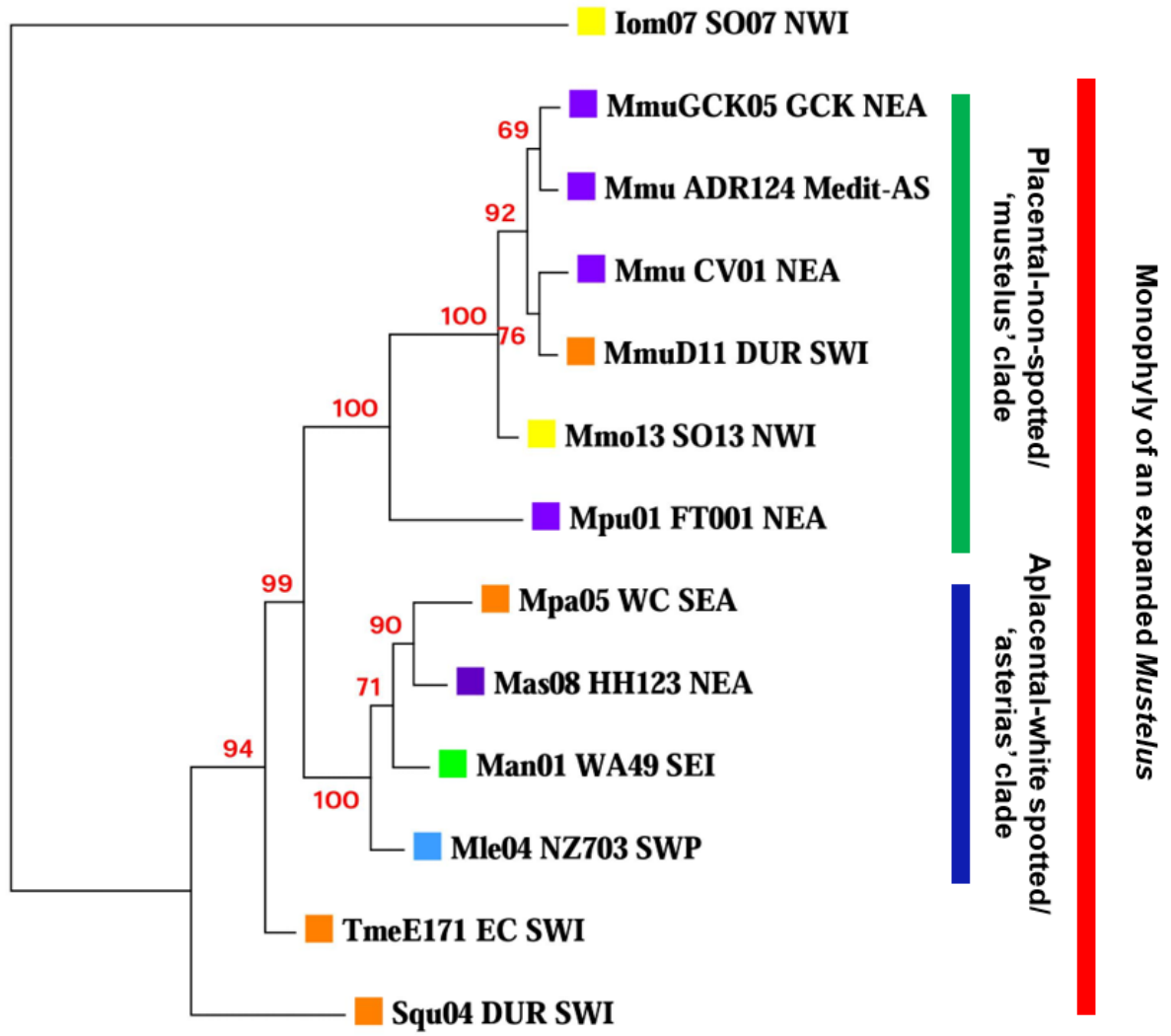

Figure 3.10 Bayesian phylogenetic hypothesis (BI) for expanded *Mustelus* based on the four gene fragments amplified. Posterior probability values are shown on the nodes. See **Table 3.1** for species codes and **Figure 3.1** for biogeographic regions.

3.4. Discussion

In the present study, the topology of the Bayesian, maximum parsimony and likelihood phylogenetic trees was largely concordant with previous phylogenies of the genus *Mustelus*, where placental non-spotted species and aplacental whitespotted species were shown to be paraphyletic groups (López *et al*. 2006; Vélez-Zuazo and Agnarsson 2011; Boomer *et al*. 2012; Naylor *et al*. 2012). In this study, the separation of the genus *Mustelus* into placental and aplacental clades was also evident with inclusion of the blackspotted smoothhound shark *M. punctulatus.* Also, *Mustelus*

formed a monophyletic group when *Scylliogaleus quecketti* and *Triakis megalopterus* were included. The blackspotted smoothhound shark was nested within the placental non-whitespotted clade and was recovered as the sister to all non-whitespotted smoothhound sharks. The basal placement of *M. punctulatus* within the placental non-whitespotted clade together with the sister relationship of *T. megalopterus* with *Mustelus* suggests the secondary loss of black spots in the genus. A number of studies report that certain populations of the common smoothhound *M. mustelus* have black spots on their dorsal surface (da Silva 2007; Marino *et al*. 2014, 2017) resembling species of the genus *Triakis*. Nevertheless, the current results support the suggestion of Boomer *et al*. (2012) that the presence or absence of white spots could give an indication of the reproductive mode of *Mustelus* especially in species in which reproduction has yet to be investigated.

It is noteworthy that the present study also uncovered relatively low levels of interspecific genetic divergence, especially within the 'aplacental' clade, indicative of recent divergence (Boomer *et al*. 2012). According to the ML analyses, some clades display a vicariant signature of range splitting ('aplacental' clade), while other clades display a geodispersal signature of synchronised range expansions ('placental' clade). Although the concept of vicariant speciation is clear and well defined, the detection and delimitation of areas of endemism is often problematic (Harold and Mooi 1994; Szumik *et al*. 2012). The dispersal of species across biogeographical barriers over time often results in sympatric species that originated in different areas of endemism (Anderson 1994). Based on the observed genetic divergence between the three southern endemics including *M. palumbes*, *S. quecketti* and *T. megalopterus*, it can be concluded that these species may have originated from different areas of endemism.

The phylogenetic hypothesis generated using the control region (CR) is perhaps the most credible from a biogeographical point of view, in spite of being single-locus analysis. The CR has been applied to evaluate genetic structure in sharks (Heist *et al*. 1996; Keeney *et al*. 2003; Chabot *et al*. 2009) and shark genetic diversification (Corrigan and Beheregaray 2009; Boomer *et al*. 2012). The suitability of the CR in providing

phylogenetic information at different levels of evolutionary divergence (intra-specific and inter-specific) is attributable to the variable substitution rates at the different CR nucleotide positions and structural domains (Heist *et al*. 1996; Keeney *et al*. 2003). In line with the earliest record of *Mustelus* in the northern hemisphere (Adnet and Cappetta 2008), the CR hypothesis strongly supported a northern hemisphere origin of southern African *Mustelus* species, where these species can be considered as remnants of the Mediterranean Sea. It is clear that the radiation of *Mustelus* in the southern African region was driven primarily by long-distance dispersal. The phylogenetic proximity of the northern endemic *M. asterias* and southern endemic *M. palumbes*, indicating recent genetic diversification of these species was also in support of this hypothesis. It is also possible that the common ancestor of *M. asterias* and *M. palumbes* was formerly more widespread across the eastern Atlantic Ocean and gradually became restricted in the northern and southern peripheries of its cosmopolitan distribution. This ''speciation event'' was most likely driven by changing habitat ecology over time or prehistoric oceanographic features, such as ocean currents and fronts. Furthermore, at the intraspecific level, sampling across multiple locations for the cosmopolitan *M. mustelus* showed apparent phylogeographic structure where the samples from the north-eastern Atlantic resided in the basal clade. The long distance dispersal ability is evident by the observation that the samples from Cape Verde were nested within the southern African clade, while the samples from Guinea-Bissau and Guinea-Conakry were in a subclade within the Mediterranean clade. Nevertheless, the paraphyly of southern African *Mustelus* species suggest that there could have been at least two separate colonisation events from the north. It is proposed that the dispersal route for the common smoothhound happened via West Africa or North-west Atlantic while for the whitespotted smoothhound the colonisation route was via the Red Sea or Indo-Pacific.

The absence of a well-calibrated molecular clock for *M. mustelus* presented a challenge in dating the time of divergence and colonization events. Estimates of mutation rates in elasmobranch taxa are only available for the noncoding

mitochondrial DNA control region (CR) (~10^{-5} mutations per generation) [scalloped hammerheads *Sphyrna lewini* (Duncan *et al*. 2006), blacktip shark *Carcharhinus limbatus* (Keeney and Heist 2006), lemon shark *Negaprion brevirostris* (Schultz *et al*. 2008)] and the mitochondrial cytochrome *b* gene (per-site divergence rate of 0.0414/million years) [bonnethead sharks *Sphyrna tiburo* (Martin *et al*. 1992), sleeper sharks subgenus *Somniosus* (Murray *et al*. 2008)]. These mutation rates have been applied to distantly related species, which were not congeneric, making the accuracy of this approach somewhat questionable (Grant *et al*. 2012, Shapiro and Ho 2014). In future further analysis of the data generated in the present study applying averaged mutation rate could allow for estimating divergence times and colonisation events.

3.5. Conclusions

In summary, the results reported in this chapter gave strong support for a northern hemisphere origin of southern African *Mustelus* species, and that the radiation of *Mustelus* in this region was primarily driven by long-distance dispersal. The monophyly of expanded *Mustelus* indicated that southern African species of the genus arose from at least two separate colonisation events from the Northern Hemisphere. A third and separate colonisation event for the isolated population of the Arabian smoothhound *M. mosis* is highly likely, and this could in future be verified if samples from the east coast of South Africa are included.

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CHAPTER 4

Comparative population genetics of two sympatric species of *Mustelus* and two coastal houndsharks

Abstract

The common smoothhound (*Mustelus mustelus*) is the topmost bio-economically and recreationally important shark species in southern Africa. Illumina HiSeq™ 2000 next-generation sequencing (NGS) technology was previously employed for *in silico* screening of novel microsatellite loci for *M. mustelus*. Here, two microsatellite multiplex panels were constructed from 11 polymorphic loci and characterised in two populations of *M. mustelus* representative of its South African distribution. The markers were tested for cross-species utility in *Galeorhinus galeus*, *M. palumbes* and *Triakis megalopterus*, three demersal coastal sharks also subjected to commercial and/or recreational fishery pressures in South Africa. Genetic diversity (N_A, A_R, H_O, H_E and *PIC*) and differentiation (F_{ST} and D_{est}) for each species were assessed and the potential use of these markers in species assignment was examined. In each of the four species, all 11 microsatellites were variable with up to a mean N_A of 8, A_R up to 7.5, H_E and *PIC* as high as 0.842. The null hypothesis of genetic homogeneity was rejected for all species investigated here except for *T. megalopterus* and although sampling was limited, descriptive and clustering analyses provide support for genetically differentiated populations associated with oceans (Atlantic and Indian) across the South-African coast. It is proposed that a combination of factors such as seascape features, movement patterns, and habitat preference explain the observed patterns of gene flow in these coastal sharks. Overall, this chapter provides molecular tools to address ecological and evolutionary questions vital to the conservation and management of these exploited shark species.

4.1. Introduction

Sharks play a crucial role in maintaining the ecological balance in marine ecosystems as keystone species, yet these animals are gradually declining worldwide in seascapes heavily impacted by humans (Dulvy *et al.* 2014). Such declines in wild populations will not only have negative ecological impacts on lower trophic species (Price *et al.* 2015) but can alter the levels and distribution of genetic diversity among populations (Dudgeon *et al.* 2012). It is likely that sharks may not respond well to population declines compared to other marine fishes owing to their *K*-selected life history traits *i.e.*, slow growth, late maturity, and low reproductive outputs (Compagno 1984; Ebert *et al.* 2013). This highlights the need for conservation and management measures to ensure the sustainable utilisation of these fisheries resources. Implementing such measures often requires information on fishery dynamics, biological and baseline ecological data which in most cases is not yet available (Velez-Zuazo *et al.* 2015). Molecular approaches have been very useful in providing insight into historical and contemporary demographies of various commercially important shark species, especially in respect to population connectivity, stock structure and metapopulation dynamics (Pereyra *et al.* 2010; Boomer 2013; Chabot *et al.* 2015; Sandoval-Castillo and Beheregaray 2015).

Despite on-going sampling difficulties, population genetics studies of bio-economically important sharks are now fast increasing due to molecular genetic markers becoming more readily available. Next-generation sequencing (NGS) and *in silico* screening of repeat loci have become a common approach to the development of microsatellites in non-model organisms (Chabot and Nigenda 2011; Blower *et al.* 2015; Pirog *et al.* 2015). Also, newly developed microsatellites for source species can be assessed for cross-species transferability in congeneric and confamilial (target) species with a relatively high success rate in elasmobranchs (Boomer and Stow 2010; Chabot 2012; Maduna *et al.* 2014; Blower *et al.* 2015; Pirog *et al.* 2015). This allows for the development of standardised panels of microsatellite multiplex PCRs useful for a

number of genetic assessments such as molecular species identification and comparative population genetics. Identification of bio-economically important sharks during port inspections is very difficult (or even impossible) when using traditional taxonomic tools because of carcass processing at sea, resulting in the loss of morphological and meristic criteria (Da Silva and Bürgener 2007; Mendonça *et al*. 2010). Several different genetic identification methods have previously been developed to resolve misidentification issues (Ward et al 2008, Blanco *et al*. 2008, Naylor *et al*. 2012) while more recently, the applicability of cross-species microsatellites for species identification have been demonstrated (Maduna *et al.* 2014; Giresi *et al.* 2015).

South Africa is an ecologically and evolutionarily dynamic region with a diverse elasmobranch fauna (Compagno 1984; Ebert *et al.* 2013; Bester-van der Merwe and Gledhill 2015) and is located in the transition zone between the Atlantic and Indo-Pacific biomes (Briggs and Bowen 2012). The Atlantic/Indian Ocean boundary in this region is characterised by two ocean basins, the South-East Atlantic Ocean (SEAO) and South-West Indian Ocean (SWIO) with two major currents, the cold Benguela Current and the warm Agulhas Current (Hutchings *et al.* 2009; Briggs and Bowen 2012). Thus far, only a few regional population genetics studies related to sharks have been conducted in southern Africa but have shed some light on the possible impact of oceanographic features on gene flow patterns of species affected by fisheries, including the tope shark (*Galeorhinus galeus*), common smoothhound (*Mustelus mustelus*), and spotted gully shark (*Triakis megalopterus*) (Bitalo *et al.* 2015; Maduna *et al.* 2016; Soekoe 2016; Bester-van der Merwe *et al.* in press). These studies showed that the interaction between the two ocean currents plays a prominent role in limiting dispersal around the southern tip of Africa, particularly in an eastward direction for the common smoothhound shark for example. Given that single-species conservation strategies do not adequately protect the biological and ecological needs of multiple species within threatened ecosystems, the focus has shifted towards multispecies approaches.

The local distribution ranges of all the triakid species (family Triakidae) investigated here, the tope shark, common smoothhound, whitespotted smoothhound (*M. palumbes*) and the spotted gully shark extends across the Atlantic/Indian Ocean boundary. This presents an ideal opportunity to test whether the interplay of oceanographic features and life history traits are the drivers of population subdivision in these sharks. The tope shark is a highly mobile semi-pelagic demersal species that is widely distributed in temperate waters (Ebert *et al.* 2013). Although sexual maturity depends on the ocean basin of origins, females reach sexual maturity at a total length (L_T) of 118–150 cm and males at 107–135 cm L_T. Reproduction is viviparous (no yolk-sac placenta) with a triennial reproductive cycle (Lucifora *et al.* 2004; McCord 2005; Ebert *et al.* 2013). Conversely, smoothhounds are relatively small and less mobile epibenthic sharks (<170 cm L_T) (Smale and Compagno 1997; Da Silva *et al.* 2013). The common smoothhound is a cosmopolitan species distributed across the Mediterranean Sea, the eastern Atlantic Ocean and the South-West Indian Ocean whereas the whitespotted smoothhound is endemic to southern Africa and is found from Namibia to northern KwaZulu-Natal (Smale and Compagno 1997; Ebert *et al.* 2013). Reproduction in the common smoothhound is characterised by placental viviparity and a seasonal reproductive cycle whereby each cycle may take one year or longer. Sexual maturity is reached at 70–112 cm L_T for males and 107.5–124 cm L_T for females (Smale and Compagno 1997; Saïdi *et al.* 2008). For the whitespotted smoothhound, reproduction is characterised by aplacental viviparity and an aseasonal reproductive cycle although the timing of reproductive cycles is presently unclear. Sexual maturity is reached at 75–85 cm L_T for males and 80–100 cm L_T for females (Smale and Compagno 1997; Ebert *et al.* 2013). Similar to smoothhounds morphologically but with a larger body size the spotted gully shark is endemic to southern Africa and is found from southern Angola to Coffee Bay, South Africa. Reproduction is ovoviviparous with a biennial to triennial reproductive cycle (Smale and Goosen 1999; Soekoe 2016). Sexual maturity is reached at 94–130 cm L_T for males and 140–150 cm L_T for females. Anecdotal evidence based on tagging data suggests

that the spotted gully sharks exhibit a high degree of site fidelity or residency since *ca.* 80% of these animals were recaptured close to their release site (within a 20 km radius), regardless of the time at liberty (Dunlop and Mann 2014; Soekoe 2016).

The project within this chapter aimed to characterise a set of *in silico* NGS mined microsatellites in the common smoothhound and evaluate the potential of cross-species utility of these markers in species identification and assessing the distribution of genetic variation across populations sampled along the South African coast.

4.2. Materials and methods

4.2.1. Sample collection and genomic DNA extraction

A total of 144 finclip samples from four coastal shark species (the tope shark, common smoothhound, whitespotted smoothhound and the spotted gully shark) were examined (**Table 4.1**). We included samples from the west and east coasts, representing the two main ocean basins (SEAO and SWIO) spanning the South African coastline (**Figure 4.1**). The west coast samples represent SEAO individuals collected west of the Atlantic/Indian Ocean boundary while the east coast samples represent SWIO individuals collected east of the Atlantic/Indian Ocean boundary. In addition, we obtained tissues samples from three individuals each of the starry smoothhound (*Mustelus asterias*) and the blackspotted smoothhound (*M. punctulatus*) from the Mediterranean Sea, and two individuals of the hardnose smoothhound (*M. mosis*) from Oman in the north-western Indian Ocean. Total genomic DNA was isolated using a standard cetyltrimethylammonium bromide (CTAB) extraction protocol of Sambrook and Russell (2001). The concentration and the quality of the extracted DNA were determined by measuring its optical density at 260 nm (A_{260}) and 280 nm (A_{280}) with a NanoDrop ND 2000 spectrophotometer (Thermo Fisher Scientific; wwwthermofisher.com). A small subset of samples was subjected to electrophoresis in 1× TAE buffer for 1 hour at 80 V. Five µL of the isolated genomic DNA was loaded on 0.8% agarose gel stained with ethidium bromide to check DNA quality. The gels

were photographed under a Gel Documentation system (Gel Doc XR+, Bio-Rad, South Africa).

Table 4.1 Details of the sampling locations and sample sizes (*N*) of four coastal shark species.

Species	Ocean basin	Collection site	Geographic co-ordinates	*N*
Mustelus mustelus	SEAO	Langebaan Lagoon	33°09′S, 18°04′E	8
(*N* = 48)		Robben Island	33°48′S, 18°24′E	8
		False Bay	34°10′S, 18°36′E	8
	SWIO	Struis Bay	34°47′S, 20°03′E	8
		Jeffreys Bay	34°35′S, 24°56′E	8
		Durban	29°44′S, 31°07′E	8
Mustelus palumbes	SEAO	Yzerfontein	33°20′S, 18°02′E	11
(*N* = 40)	SWIO	Mossel Bay	34°09′S, 22°10′E	13
	Unknown	-	-	16
Galeorhinus galeus	SEAO	Robben Island	33°48′S, 18°24′E	7
(*N* = 24)		False Bay	34°10′S, 18°36′E	7
	SWIO	Struis Bay	34°47′S, 20°03′E	3
		Mossel Bay	34°09′S, 22°10′E	2
		Port Elizabeth	34°04′S, 25°03′E	5
Triakis megalopterus	SEAO	Cape Point	34°20′S, 18°33′E	8
(*N* = 32)		Betty's Bay	34°22′S, 18°55′E	8
	SWIO	Port Elizabeth	34°04′S, 25°03′E	16

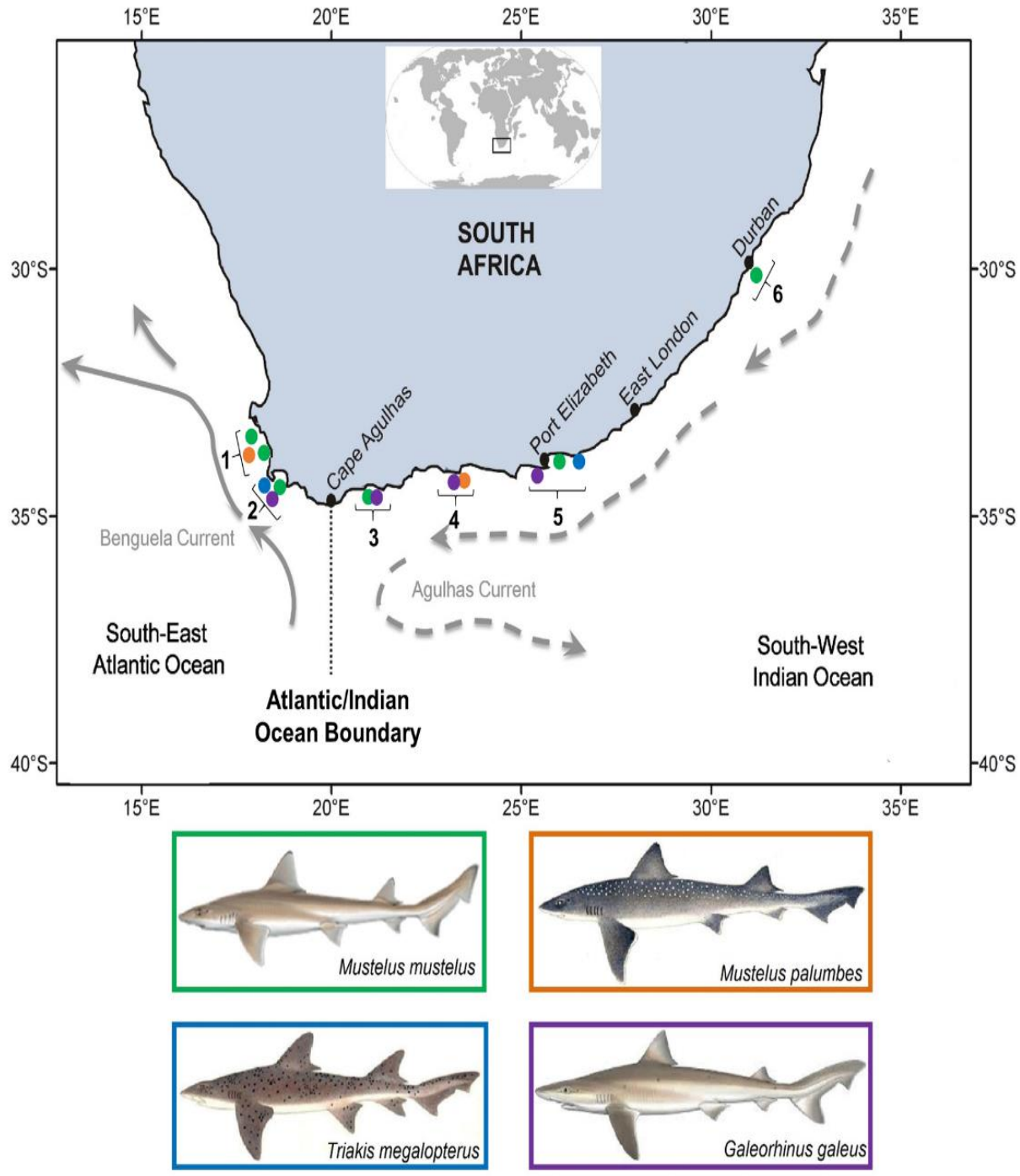

Figure 4.1 Sampling localities of four coastal shark species with the green circle representing *Mustelus mustelus*, and orange, blue and purple circles representing *Mustelus palumbes*, *Triakis megalopterus* and *Galeorhinus galeus*, respectively. Locations 1-2 and 3-6 represent the South African South-East Atlantic and South-West Indian Ocean sampled populations, respectively. The major oceanographic features are also shown.

4.2.2. Development of species-specific microsatellites

Total genomic DNA from one individual of common smoothhound was isolated and sent to the Agricultural Research Council Biotechnology Platform in Pretoria, South Africa. One μg of genomic DNA was used for 2 × 250 bp paired-end library preparation with a mean insert size of 400 bp using the standard Illumina Nextera™ library preparation kit. The library was sequenced on two lanes of an Illumina HiSeq™ 2000 sequencer. The generated sequence reads were submitted to a quality control (QC) step to remove artificial duplicates and/or reads that contained any 'Ns' using PRINSEQ v. 0.20.4 (Schmieder and Edwards 2011). Reads were quality-filtered and trimmed to remove all Nextera adapters and sequences shorter than 35 bp using TRIMMOMATIC v. 0.33 (Bolger *et al*. 2014) with default settings. A phred quality score of 15 was selected and sequences that contained at least 90% of the individual bases above this quality score were filtered.

To check whether primer, barcode, and adapter sequences have been properly trimmed, sequencing quality was visualized using the software FASTQC v. 0.11.4 (Andrews 2010). After the QC step, contigs were built from processed read files using ABYSS v. 1.5.2 (Simpson *et al*. 2009) and contigs larger than 250 bp were selected for microsatellite identification in MISA v. 1.0 (Thiel *et al*. 2003). Sequences with ≥ 5 uninterrupted motifs towards the middle were selected and blasted against the NCBI database to filter for the contigs which contained hits with microsatellites against other elasmobranch or teleost species. Sequences with hits were selected for primer design using PRIMER3 v. 0.4.0 (Untergrasser *et al*. 2012).

4.2.3. Microsatellite validation and transferability

Polymerase chain reaction (PCR) was carried out on a GeneAmp® PCR System 2700 in a 10 μL reaction volume that included 50 ng of template DNA, 1x PCR Buffer, 200 μM of each dNTP, 0.2 μM of each primer, 1.5 mM $MgCl_2$ and 0.1 U of GoTaq® DNA polymerase. The PCR cycling conditions were as follows (i) one cycle of initial

denaturation at 95°C for 2 min, (ii) 35 cycles of denaturation at 94°C for 30 s, optimised annealing temperature (T_A) for 30 s, elongation at 72°C for 2 min, (iii) a final elongation of one cycle at 60°C for 5 min and thereafter stored at 4°C. Optimum annealing temperature was determined by experimental standardisation for each of the primer pairs (**Table 4.2**). Amplification products were subjected to agarose gel electrophoresis to determine their size.

Levels of polymorphism were initially assessed at all the successfully amplified microsatellite loci in a panel of eight individuals of *M. mustelus*. The amplified PCR products were resolved on a vertical non-denaturing 12% polyacrylamide gel to detect size variants. Microsatellites were designated polymorphic when two bands were distinguishable in a single individual (*i.e.* heterozygote) and/or we observed clear size differences between different individuals. Polymorphic microsatellite loci were selected and primers fluorescently labelled with one of the following dyes: FAM, VIC, PET, or NED followed by multiplex optimisation of two mutiplex assays (MPS1 and MPS2).

A panel of 48 individual *M. mustelus* representative of the two ocean basins (SEAO and SWIO) was genotyped for marker characterisation. Multiplex PCR conditions were realized using the Qiagen Multiplex PCR kit and conducted according to the manufacturer's instructions except for varying primer concentrations (**Table 4.3**) and T_A, 56°C for MPS1 and 57°C for MPS2. For subsequent analysis on an ABI 3730XL DNA Analyzer, PCR products were diluted in distilled water and fragment analysis performed together with the LIZ600 internal size standard. Individual genotypes were scored based on fragment size via GENEMAPPER v. 4.0 (Life Technologies, South Africa).

To determine the utility of these markers for future regional studies of intra- and interspecific genetic diversities in houndsharks (Triakidae), we also tested the 11 microsatellite loci on the blackspotted smoothhound, spotted gully shark, starry smoothhound, tope shark and whitespotted smoothhound using the PCR reactions and microsatellite genotyping conditions described above.

4.2.4. Microsatellite characterisation

For the four study species, all loci were assed for scoring errors and allelic dropout using MICROCHECKER v. 2.2.3 (Van Oosterhout *et al*. 2004). The Microsatellite Excel Toolkit (MSATTOOLS v. 1.0, Park 2001) was used to identify samples sharing identical multilocus genotypes. Duplicate genotypes with ≥ 95% matching alleles were excluded from further analyses.

Using FREENA (Chapuis and Estoup 2007), the frequency of null alleles were estimated following the Expectation Maximization (EM) method described by Dempster *et al*. (1977). Deviations from Hardy–Weinberg equilibrium (HWE) for each locus was evaluated using the exact probability test based on 10 000 iterations (10 000 dememorisation, 500 batches) in GENEPOP v. 4.0 (Rousset, 2008). Linkage disequilibrium among loci was assessed using an exact test, also implemented in GENEPOP.

False discovery rate (FDR; Benjamini and Yekutieli 2001) control was used to adjust *P*-values for multiple comparisons (*i.e*. tests for departure from HWE and linkage disequilibrium) to minimize type I errors (see Narum 2006). To test for potential signatures of selection for each locus LOSITAN v. 1.44 (Antao et al. 2008) with 200 000 simulations following the F_{ST}-outlier method of Beaumont & Nichols (1996) we used.

Table 4.2 Details of 15 microsatellite loci developed for *Mustelus mustelus*, where F = Forward, R = Reverse and T_A = annealing temperature in °C

Locus name	Primer sequence	Motif	PCR Product size	TA (°C)	BLAST hit	PAGE results	Accession number
Mmu1	F-CCCCATTTGCAAACAGAGTT	$(AT)_7$	210	57	*Danio rerio*	Polymorphic	KX261856
	R-ATTTCCCGCTGTTACATTGC						
Mmu2	F-TTGTCTGCAGGAAACACAGC	$(AC)_6$	163	56	*Cyprinus carpio*	Polymorphic	KX261857
	R-GCATCGTGTGAAATGGGAAT						
Mmu3	F-ATACACGGACCGACTCGAAC	$(TC)_7$	240	56	*Astyanax mexicanus*	Polymorphic	KX261858
	R-TAATGCCGAGATCAGGAACC						
Mmu4	F-TCCATCCAGCGTTAAAGGAC	$(TG)_7$	173	56	*Astyanax mexicanus*	Polymorphic	KX261859
	R-GCACCAGAGCTTCCCATTTA						
Mmu5	F-ACCACTCCCTGCAGCACTAC	$(CTC)_6$	282	57	*Callorhinchus milii*	Polymorphic	KX261860
	R-AGGAGATGCTTTGGCACTTG						
Mmu6	F-CACCGGAGACCTCTAACTGG	$(CGC)_6$	212	57	*Chrysemys picta bellii*	Polymorphic	KX261861
	R-CGATGATGATGAAGGACGTG						
Mmu7	F-TCCCTCATTTGCTTCAGGAG	$(GCT)_5$	219	57	*Callorhinchus milii*	Polymorphic	KX261862
	R-CGACATGAAACGCAGAAAGA						
Mmu8	F-AGTAAGGCGCGCTATGATTG	$(CAG)_5(TGT)_5$	431	56	*Callorhinchus milii*	Polymorphic	KX261863
	R-TAGAAGTCATCGCCCTCCAC						

Table 4.2 Continued.

Locus name	Primer sequence	Motif	PCR Product size	TA (°C)	BLAST hit	PAGE results	Accession number
Mmu9	F-ACGGTTCTGAGCAATCGTCT R-TGCGATATTCGTCAGGTGAA	$(GAAT)_5$	172	56	*Callorhinchus milii*	Monomorphic	KX261864
Mmu10	F-AATCCTGAGCACCAGGACAC R-TGTGTGAATTCCCCAGATGA	$(CATA)_5$	299	56	*Squalus acanthias*	Monomorphic	KX261865
Mmu11	F-ATCTTGTTAACCGCCGACAG R-CGCCATGTTGATCGAAGTAA	$(CAA)_5$	211	56	*Callorhinchus milii*	Polymorphic	KX261866
Mmu12	F-GAGCAGCCAAGCATTAGTCC R-CGGCTTCAGAAATTGGAATC	$(GAT)_6$	208	56	*Callorhinchus milii*	Monomorphic	KX261867
Mmu13	F-TCATTCCTCACACCCACTCA R-AGATCCAGGAGCGAAGAACA	$(GCA)_5$	112	56	*Squalus acanthias*	Polymorphic	KX261868
Mmu14	F-ACCGCTTGCTTCTGTTGAGT R-TCGCACAGACTGATTGAAGG	$(AGC)_6$	186	58	*Callorhinchus milii*	Polymorphic	KX261869
Mmu15	F-CACCTGATTGAGCAGGAGGT R-TATGGAGGTTGGGATTGCAG	$(CTC)_5$	173	58	*Squalus acanthias*	Monomorphic	KX261870

4.2.5. Within species population genetic analysis

Across sampling sites and species, the mean number of alleles per locus (N_A), allelic richness standardized for small sample size (A_R), observed heterozygosity (H_O) and heterozygosity expected under conditions of Hardy–Weinberg equilibrium (H_E) were calculated using the DIVERSITY (Keenan *et al.* 2013) package for R (R Development Core Team 2015). MSATTOOLS was used to calculate the polymorphic information content (*PIC*) according to the equation described in Botstein *et al*. (1980). The inbreeding coefficient (F_{IS}) was calculated in ARLEQUIN v. 3.5 (Excoffier and Lischer 2010) and tested for deviations from zero using a permutation test (1000 permutations) with significance values adjusted using the FDR correction for multiple tests. Subsequently, POWSIM v. 4.1 (Ryman and Palm 2006) was employed to assess the statistical power of the loci for F_{ST} tests (*i.e*. rejection of the null hypothesis H_0 of genetic homogeneity among two subpopulations when it is false) and the α level (*i.e*. rejection of H_0 when it is true) using a sampling scheme of two subpopulations with 20 individuals each. The analyses were conducted using 10 000 dememorizations, 100 batches, and 1000 iterations per batch with the allele frequencies observed for the complete dataset of 11 microsatellite loci and our reported sample sizes for each species.

Pairwise F_{ST} (Weir and Cockerham 1984) and Jost's D_{est} (Jost, 2008) were calculated using the DIVERSITY package, and the analysis of molecular variance (AMOVA) was calculated using ARLEQUIN. To account for the study sampling strategy, the measures of genetic differentiation comparisons were considered significantly positive if the lower limit of CI was > 0, and *P*-values were < 0.05 following FDR correction. To visualise population distinctness, ADEGENET was used to perform discriminant analysis of principal components (DAPC) on clusters defined by ocean basin. The number of clusters was assessed using the *find.clusters* function, which runs successive *K*-means clustering with increasing number of clusters (*k*). For selecting the optimal *k*, the Bayesian Information Criterion (BIC) for assessing the best supported model was applied, and therefore the number and nature of clusters, as recommended

by Jombart *et al*. (2010). Discriminant analysis of principal components scatter plots were only drawn for $k > 2$.

A Bayesian clustering model-based method implemented in STRUCTURE 2.3 (Pritchard *et al*. 2000) was also used to detect the most probable number of genetic clusters (K) present in each species. An admixture model with correlated allele frequencies was applied for 10 replicates across $K = 1$ to $K = 10$ with each run consisting of 1 000 000 Markov Chain Monte Carlo (MCMC) iterations and an initial burn-in phase of 100 000 iterations assuming no prior population information. Given that only two groups of samples were compared for each species, the *ad hoc* statistic ΔK described in Evanno *et al.* (2005) and commonly used to identify the likely number of genetic clusters, was not considered appropriate for our study. This ΔK statistic never assigns $K = 1$ (Evanno *et al.* 2005). Here, the posterior probability of the data (X) for a given K, $\Pr(X|K)$, calculated by STRUCTURE was used to compute the mean likelihood $L(K)$ over 10 runs for each K to identify the likely K for which $L(K)$ was highest (Pritchard *et al*. 2000) as implemented in STRUCTURE HARVESTER 0.6.94 (Earl and vonHoldt 2012). CLUMPAK (Kopelman *et al*. 2015) was used for the graphical representations of the STRUCTURE results.

Given that there was uncertain about sampling locations of several individual *Mustelus palumbes,* the program GENECLASS2 v2.0 (Piry *et al*. 2004) was also used to examine genetic structure based on assignment tests for this species. Assignment probabilities of individuals were calculated using a Bayesian procedure (Rannala and Mountain 1997) and Monte Carlo re-sampling using 100 000 simulated individuals and a threshold of 0.01.

Finally, the coalescence-based method in the program MIGRATE-N 3.6.11 (Beerli 2006, Beerli and Palczewski 2010) implemented on the CIPRES Portal v3.3 at the San Diego Supercomputer Center (Miller *et al*. 2010) was used to compare alternative migration pattern across oceans. Four migration models were assessed: (1) a full model with two population sizes and two migration rates (from SEAO to SWIO and from SWIO to SEAO); (2) a model with two population sizes and one migration rate

to SEAO; (3) a model with two population sizes and one migration rate to SWIO; (4) a model where SEAO and SWIO are part of the same panmictic population. The mutation-scaled effective population size $\Theta = 4N_e\mu$, where N_e is the effective population size and μ is the mutation rate per generation per locus, as well as mutation-scaled migration rates $M = m/\mu$, where m is the immigration rate per generation among populations were also calculated in MIGRATE-N. A Brownian process was used to model microsatellite mutations. The Metropolis–Hastings algorithm was used to sample from the prior distributions and generate posterior distributions. Each model was run using random genealogy and values of the parameters Θ and M produced by F_{ST} calculation as a start condition. Bayesian search strategy was conducted using the following parameters: an MCMC search of 5×10^5 burn-in steps followed by 5×10^6 steps with parameters recorded every 20 steps. The prior distribution for the parameters was uniform with Θ and migration boundaries defined after explorative runs. A static heating scheme with four different temperatures (1.0, 1.5, 3.0, 1×10^6) was employed, where acceptance–rejection swaps were proposed at every step. The model comparison was made using log-equivalent Bayes factors (LBF) that need the accurate calculation of marginal likelihoods. These likelihoods were calculated using thermodynamic integration in MIGRATE-N. Models were ordered by LBF, and the model probability (P_{Mi}) was calculated in R. Additionally, we converted estimates of gene flow (M) to the number of effective migrants (N_em) from population i to population j using the formula:

$$N_e^{(j)} m_{i \to j} = \frac{\Theta_j M_{i \to j}}{4}$$

4.3. Results

4.3.1. Microsatellite multiplex assays cross-species transferability

The two sequencing runs of the Nextera™ library for *Mustelus mustelus* generated 35 GB of raw reads. After trimming the raw sequences that included removal of adapters, N-containing reads, and low-quality reads, a total of 17 GB clean reads were retained. After the *de novo* assembly of the Illumina paired-end reads, a total of 27 512 666 contigs were recovered. A total of 82 879 contigs that were longer than 250 bp were identified, of which 2 572 (3.1%) contained microsatellites. Dinucleotide repeats were the most frequent (1 629, or 86.1%), followed by trinucleotide repeats (232, or 12.3%), and tetranucleotide repeats (31, or 1.6%). A total of 15 microsatellite containing contigs were selected for primers design with an expected PCR product size ranging between 112 bp and 431 bp. Out of the 15 loci tested, all were successfully amplified while only 11 were polymorphic based on initial screening via polyacrylamide gels (**Table 4.2**). These loci were fluorescently labelled to construct a 5-plex and 6-plex assay that were both validated over 48 individuals from two populations of the common smoothhound (**Figures A4.1** and **A4.2**, **Appendix**). The genetic diversity summary statistics for both multiplex assays are presented in **Table 4.3**. All loci were polymorphic and produced a total of 74 alleles (mean 6.2). There was no evidence of stutter products or significant allelic dropout based on the MICRO-CHECKER results, but null alleles were detected at two loci (Mmu5 and Mmu14) with high frequencies estimated in FREENA relative to the rest of the loci (**Table 4.3**). After correcting for multiple tests, all loci agreed with HWE except for Mmu5 and Mmu14 possibly due to null alleles. Linkage disequilibrium was not found between any of the loci pairs tested. The F_{ST}-outlier test showed that locus Mmu7 did not conform to selective neutrality and was under putative directional selection. The *PIC* ranged from 0.08 to 0.76 and the H_O and H_E ranged from 0.09 to 1 and 0.08 to 0.79, respectively. The F_{IS} value ranged from -0.506 to 0.759. Subsequent estimates of population genetic structure were therefore computed using a subset of eight microsatellites, excluding loci not conforming to Hardy-Weinberg equilibrium, neutrality, and/or exhibiting high null allele frequencies (Mmu5, Mmu7 and Mmu14).

Table 4.3 Characteristics of two polymorphic microsatellite multiplex assays for *Mustelus mustelus* based on two sampling ocean basins in South Africa, Southeast Atlantic Ocean (SEAO) and South-West Indian Ocean (SWIO). Primer concentration in the final reaction as μM/primer ([P]); Number of individuals (*N*), Number of alleles per locus (N_A); allelic richness (A_R); observed heterozygosity (H_O); expected heterozygosity (H_E); polymorphic information content (*PIC*); Probability of conformity to Hardy-Weinberg expectations (P_{HW}); null allele frequency (Fr_{NULL}). Mean values for each multiplex assay and overall are indicated in bold.

Locus	Microsatellite repeat motif	[P]	Dye	Ocean basin	*N*	Size range (bp)	N_A	A_R	H_O	H_E	*PIC*	F_{IS}	P_{HW}	Fr_{NULL}
Mmu2	$(AC)_6$	0.2	FAM	SEAO	25	150-180	5	4.8	0.96	0.68	0.63	-0.391	0.000	0.000
				SWIO	23		4	3.5	0.65	0.48	0.41	-0.352	0.495	0.000
Mmu3	$(TC)_7$	0.2	NED	SEAO	25	230-250	4	3.6	0.48	0.54	0.48	0.127	0.643	0.005
				SWIO	22		6	5.2	0.41	0.65	0.59	0.393	0.054	0.121
Mmu4	$(TG)_7$	0.2	VIC	SEAO	25	158-180	8	6.3	0.88	0.67	0.61	-0.297	0.000	0.025
				SWIO	23		4	3.5	0.61	0.48	0.41	-0.260	0.368	0.000
Mmu8	$(CAG)_5(TGT)_5$	0.3	VIC	SEAO	25	417-440	8	7.1	0.72	0.78	0.75	0.096	0.710	0.016
				SWIO	21		8	6.8	0.62	0.79	0.76	0.240	0.416	0.094
Mmu11	$(CAA)_5$	0.3	PET	SEAO	24	203-209	3	3.0	0.75	0.62	0.55	-0.181	0.033	0.000
				SWIO	16		4	3.9	0.69	0.69	0.63	0.035	0.352	0.000
Mmu13	$(GCA)_5$	0.2	NED	SEAO	25	85-109	4	3.6	0.96	0.63	0.57	-0.506	0.001	0.000
				SWIO	23		5	4.5	0.78	0.57	0.53	-0.349	0.485	0.000
MPS1 (mean)	**-**	**-**	**-**			**-**	**6.5**	**5.6**	**0.714**	**0.651**	**0.588**	**-0.097**	**-**	**0.027**

Table 4.3 Continued.

Locus	Microsatellite repeat motif	[P]	Dye	Ocean basin	*N*	Size range (bp)	N_A	A_R	H_O	H_E	*PIC*	F_{IS}	P_{HW}	Fr_{NULL}
Mmu1	$(AT)_7$	0.2	VIC	SEAO	24	200-216	5	4.9	1.00	0.69	0.64	-0.428	0.072	0.000
				SWIO	24		6	5.2	0.92	0.66	0.62	-0.369	0.443	0.000
Mmu5	$(CTC)_6$	0.3	FAM	SEAO	24	268-274	2	2.0	0.12	0.50	0.37	0.759	0.000	0.250
				SWIO	22		4	3.2	0.23	0.53	0.43	0.590	0.067	0.193
Mmu6	$(CGC)_6$	0.3	PET	SEAO	24	204-214	5	4.2	0.38	0.36	0.33	-0.035	0.000	0.000
				SWIO	21		5	4.5	0.62	0.59	0.55	-0.022	0.040	0.020
Mmu7	$(GCT)_5$	0.2	NED	SEAO	23	203-217	3	2.2	0.09	0.08	0.08	-0.011	0.997	0.000
				SWIO	24		6	5.2	0.50	0.52	0.49	0.061	0.143	0.000
Mmu14	$(AGC)_6$	0.2	FAM	SEAO	24	160-180	5	4.6	0.38	0.70	0.64	0.480	0.003	0.189
				SWIO	24		5	4.6	0.50	0.70	0.65	0.310	0.008	0.114
MPS2 (mean)	**-**	**-**	**-**			**-**	**5.8**	**4.9**	**0.471**	**0.554**	**0.496**	**0.150**	**-**	**0.078**
Overall (mean)	**-**	**-**	**-**			**-**	**6.2**	**5.3**	**0.604**	**0.607**	**0.546**	**0.005**	**-**	**0.050**

To assess the cross-species utility of the two multiplexes, these assays were tested on six other triakid species, and cross-species amplification rate of success ranged from 72% to 100% (**Table 4.4**). Additionally, to validate the potential of these markers for within species population genetic analysis, genetic variation was inferred in samples collected from two different ocean basins for each respective species (**Table 4.1**). In each species, all 11 microsatellites were variable with up to a mean N_A of 8, A_R up to 7.5, H_E and *PIC* as high as 0.842 (**Table A4.1**, **A4.2** and **A4.3**). After correcting for multiple tests, all loci in each species conformed to HWE and no evidence for LD between any of the loci pairs were found. MICRO-CHECKER indicated the presence of null alleles at locus Mmu11 for the tope shark and locus Mmu4 for the spotted gully shark. Using the F_{ST}-outlier test evidence for deviations from neutrality was only found in two loci (Mmu 2 and Mmu11) in the whitespotted smoothhound possibly due to issues surrounding small sample sizes. Assessment of the power of the multilocus dataset to detect population structure indicated that all loci used could accurately detect differentiation as low as F_{ST} = 0.003, for a population sample of n = 20, indicating that the dataset was suitable for population structure inference.

4.3.2. Population genetic structure and gene flow

4.3.2.1. Common smoothhound *Mustelus mustelus*

The pairwise population differentiation indices (F_{ST} = 0.029, D_{est} = 0.021) and AMOVA (F_{ST} = 0.029, **Table S4.4**) indicated the presence of shallow population genetic structure between SEAO and SWIO (*i.e.*, lower limit of 95% confidence intervals >0, and *P*-values <0.05 after FDR corrections). The DAPC analysis including location prior revealed two clear genetic clusters corresponding to ocean basins, whereas excluding location prior using the *find.clusters* function, the DAPC analysis identified the presence of five genetic clusters (k = 5) in the dataset based on the BIC score (**Figure 4.2**). The post processing of the STRUCTURE results using $L(K)$ revealed one admixed cluster (K = 1) as the most likely number of groups present in the dataset (**Figures A4.3a** and **A4.4a**). Coalescent analyses for migration model comparison highly

Table 4.4 Cross-species transfer results of 11 microsatellites tested among six triakid species, where – = no visible band or faint bands with insufficient band intensity for scoring alleles were observed, + = solid bands with sufficient intensity for scoring alleles were detected and in brackets the number of alleles per locus are shown.

Locus/Species	*Galeorhinus galeus*	*Mustelus asterias*	*M. mosis*	*M. palumbes*	*M. punctulatus*	*Triakis megalopterus*
	(N = 8)	(N = 3)	(N = 2)	(N = 8)	(N = 3)	(N = 8)
Mmu1	+ (3)	+ (1)	+ (4)	+ (3)	+ (4)	+ (2)
Mmu2	+ (3)	+ (2)	+ (1)	+ (2)	+ (1)	+ (2)
Mmu3	+ (2)	+ (3)	+ (3)	+ (2)	+ (3)	+ (2)
Mmu4	+ (2)	+ (4)	+ (2)	+ (3)	+ (4)	+ (2)
Mmu5	+ (2)	+ (2)	+ (1)	+ (2)	+ (2)	+ (2)
Mmu6	+ (2)	+ (1)	+ (1)	+ (2)	-	+ (2)
Mmu7	+ (2)	+ (1)	+ (2)	+ (3)	-	+ (2)
Mmu8	+ (4)	+ (2)	+ (1)	+ (5)	+ (2)	+ (2)
Mmu11	+ (2)	+ (1)	+ (1)	+ (2)	+ (3)	+ (2)
Mmu13	+ (3)	+ (2)	+ (2)	+ (3)	+ (6)	+ (3)
Mmu14	+ (2)	+ (2)	+ (3)	+ (2)	-	+ (2)

supported (P_{Mi} = 1.0) Model 2 (*i.e*. migration from SWIO to SEAO) and showed that Θ was highest in the SWIO (Θ = 5.870) and lowest in the SEAO (Θ = 0.790) (**Tables A4.5** and **A4.6**).

4.3.2.2. Whitespotted smoothhound *Mustelus palumbes*

Pairwise differentiation tests using F_{ST} indicated significant population differentiation estimates, which were congruent with the results obtained with Jost's D_{est} between all putative populations. Pairwise comparisons of the unknown samples (in terms of sampling region) with the samples collected from the SEAO revealed low differentiation (F_{ST} = 0.021, D_{est} = 0.017, lower 95% CI > 0), higher levels when compared with the SWIO samples (F_{ST} = 0.086, D_{est} = 0.104, lower 95% CI > 0). Notably, population differentiation estimates were significantly large for Atlantic versus Indian Ocean comparisons (F_{ST} = 0.091, D_{est} = 0.155, lower 95% CI > 0). Global AMOVA results indicated within-individual variation explains a greater amount of the total genetic variation, with less variation among populations (F_{ST} = 0.069, P < 0.01) (**Table A4.4**). The DAPC analysis including and excluding the location prior revealed three genetic clusters (k = 3) in the dataset based on the BIC score (**Figure 4.3**). Individual assignment test based on a Bayesian approach for mapping the origin of the unknown putative population assigned 60% of the individuals to the SEAO and the remainder to the SWIO, indicative of the possible existence of substructure in *M. palumbes*. Bayesian clustering analysis in STRUCTURE also supported the assignment of the unknown population to the SEAO and interoceanic population subdivision (**Figures A4.3b** and **A4.4b**). The most likely number of groups present in the data was K = 3. All results considered, we assumed the unknown putative population to have been sampled from the SEAO, therefore, for the gene flow analysis, we grouped the unknown samples with the samples from the SEAO. The most probable Migrate-N coalescent model of population structure was the unidirectional model assuming asymmetric migration from SWIO to SEAO (P_{Mi} = 1.0). Estimates of Θ was highest in the SWIO (Θ = 19.660) and lowest in the SEAO (Θ = 0.540) (**Table A4.5** and **A4.6**).

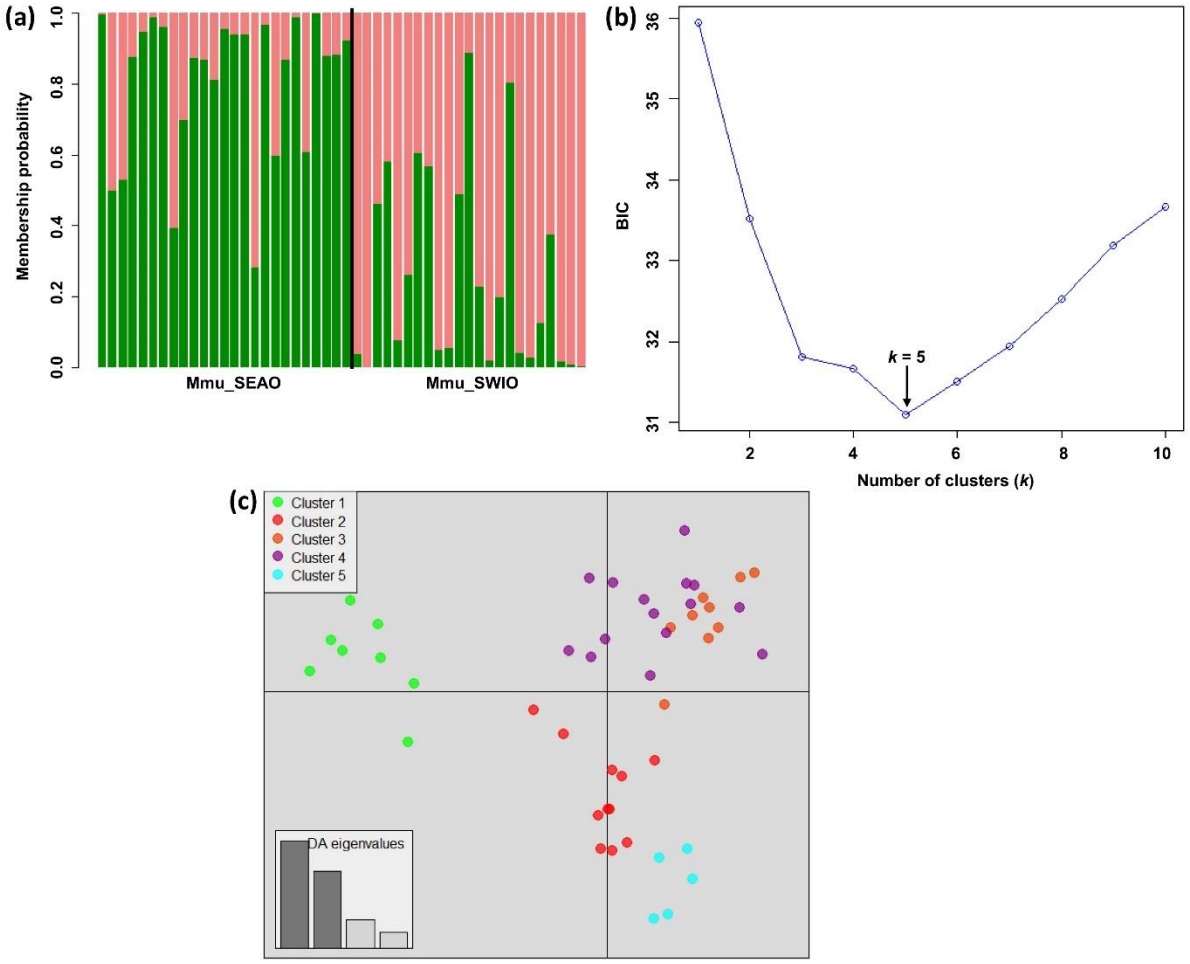

Figure 4.2 STRUCTURE-like plot, inference of the number of clusters and scatterplots of DAPC analysis on the dataset of *Mustelus mustelus*. Mmu_SEAO and Mmu_SWIO represents the South African South-East Atlantic and South-West Indian Ocean sampled populations, respectively. (a) Cluster assignments by population (sampling location *a priori*), each individual is represented by a vertical coloured line. (b) Inference of the number of clusters excluding sampling location as *a priori*. A *k* value of 5 (the lowest BIC value) represents the best summary of the data. (c) The graph represents the individuals as dots. Each colour represents a genetic cluster (*k*).

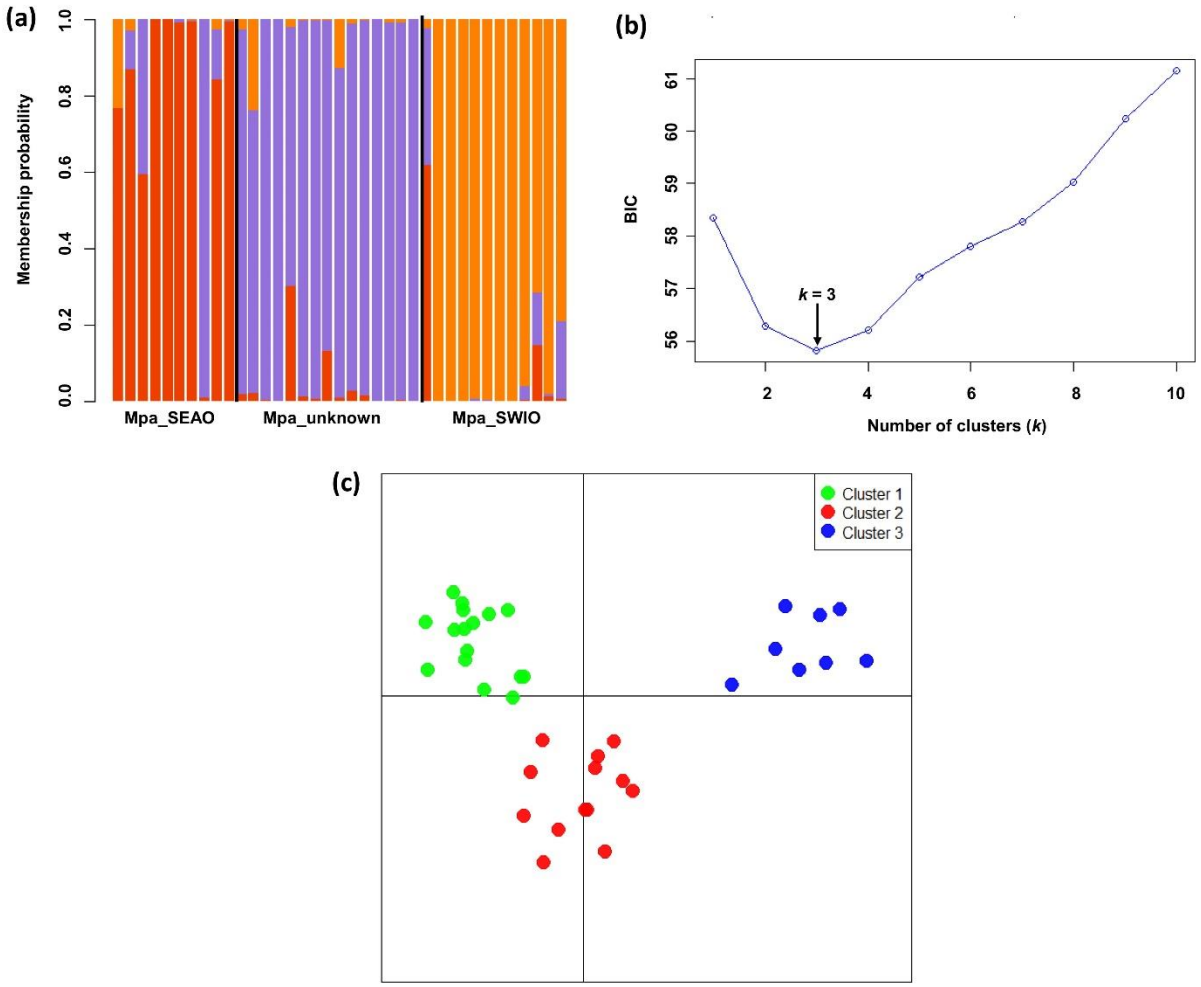

Figure 4.3 STRUCTURE-like plot, inference of the number of clusters and scatterplots of DAPC analysis on the dataset of *Mustelus palumbes*. Mpa_SEAO and Mpa_SWIO represents the South African South-East Atlantic and South-West Indian Ocean sampled populations, respectively. (a) Cluster assignments by population (sampling location *a priori*), each individual is represented by a vertical coloured line. (b) Inference of the number of clusters excluding sampling location as *a priori*. A *k* value of 3 (the lowest BIC value) represents the best summary of the data. (c) The graph represents the individuals as dots. Each colour represents a genetic cluster (*k*).

4.3.2.3. Tope shark *Galeorhinus galeus*

Population differentiation between the SEAO and SWIO was significantly greater than zero (F_{ST} = 0.034, lower 95% CI > 0), while similar to *M. mustelus*, Jost's D_{est} indicated less pronounced levels of differentiation (D_{est} = 0.076, lower 95% CI > 0). The AMOVA results showed that there was no differentiation among populations (F_{ST} = 0.033, P = 0.135), but a significant amount of variance was attributed to among individuals within populations (F_{IS} = 0.093, P = 0.000) and within individuals (F_{IT} = 0.123, P = 0.000) (**Table A4.4**). The DAPC analysis including and excluding the location prior revealed two genetic clusters (k = 2) in the dataset based on the BIC score (**Figure 4.4**). Evaluation of the K values produced by STRUCTURE using the maximum value of $L(K)$ identified K = 2 as the most likely number of groups present in the data (**Figures A4.3c** and **A4.4c**). Coalescent analyses for migration model comparison highly supported (P_{Mi} = 1.0) Model 2 (i.e. migration from SWIO to SEAO) and showed that Θ was highest in the SWIO (Θ = 98.100) and lowest in the SEAO (Θ = 0.100) (**Tables A4.5** and **A4.6**).

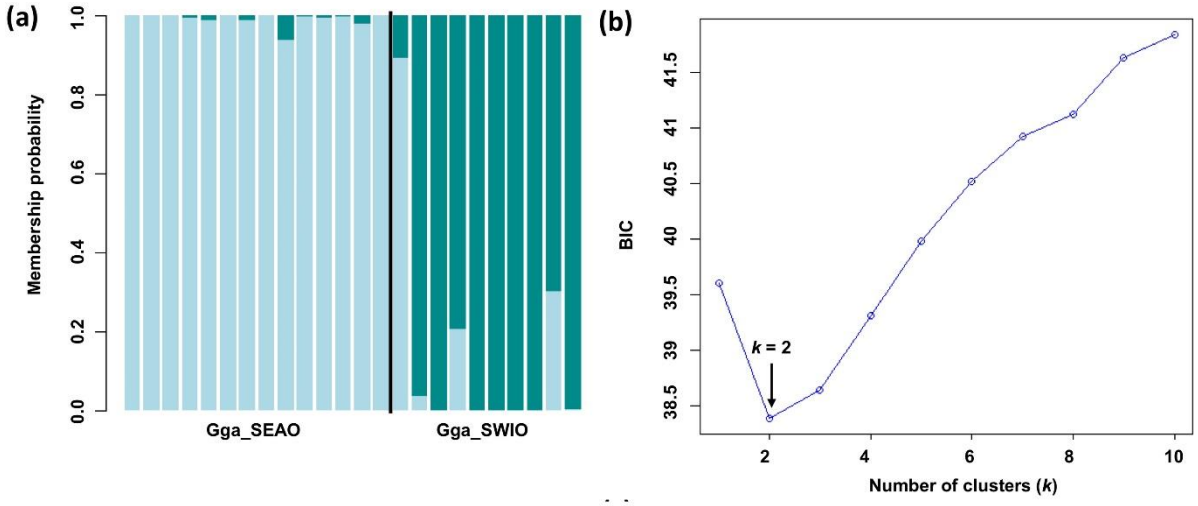

Figure 4.4 STRUCTURE-like plot, inference of the number of clusters and scatterplots of DAPC analysis on the dataset of *Galeorhinus galeus*. Gga_SEAO and Gga_SWIO represents the South African South-East Atlantic and South-West Indian Ocean sampled populations, respectively. (a) Cluster assignments by population (sampling location *a priori*), each individual is represented by a vertical coloured line. (b) Inference of the number of clusters excluding sampling location as *a priori*. A k value of 2 (the lowest BIC value) represents the best summary of the data. Each colour represents a genetic cluster (k).

4.3.2.4. Spotted gully shark *Triakis megalopterus*

Based on the population differentiation estimates there was no evidence for population subdivision between the SEAO and SWIO samples (F_{ST} = -0.012, D_{est} = -0.002, upper 95% CI > 0). The AMOVA results also showed no differentiation among populations (F_{ST} = -0.012, P = 1.000), with most of the variation explained among individuals within populations (F_{IS} = 0.134, P = 0.000) and within individuals (F_{IT} = 0.123, P = 0.000) (**Table A4.4**). The DAPC analysis showed clustering with fairly flat distributions of membership probabilities of individuals across clusters indicative of one genetic cluster in the data (**Figure 4.5**). Bayesian clustering analysis in STRUCTURE identified four admixed genetic clusters (K = 4) as the most likely number of groups present in the data (**Figure A4.3d** and **A4.4d**). Coalescent analyses for migration model comparison highly supported (P_{Mi} = 1.0) Model 2 (i.e. migration from SWIO to SEAO) and showed that Θ was highest in the SWIO (Θ = 6.820) and lowest in the SEAO (Θ = 1.380) (**Table A4.5** and **A4.6**).

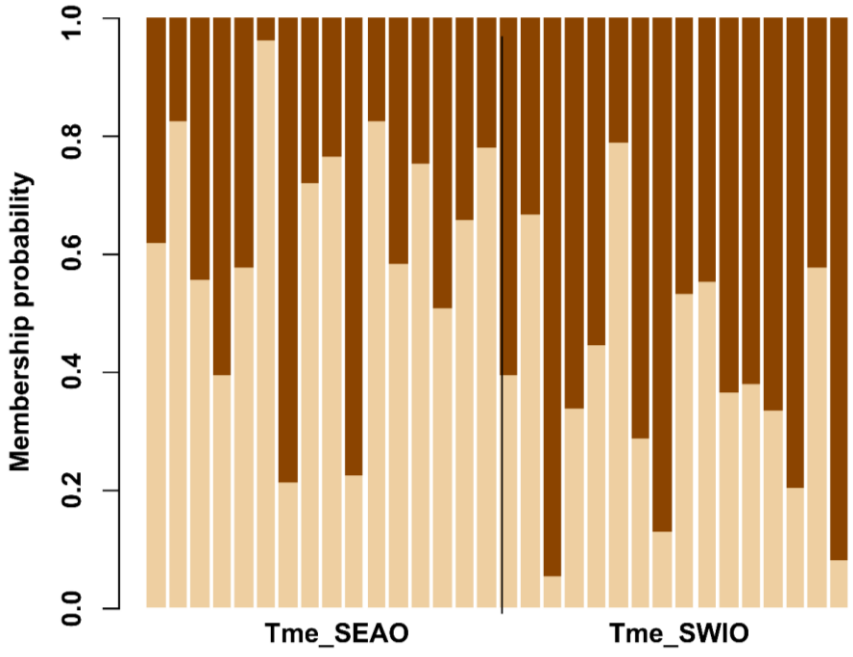

Figure 4.5 STRUCTURE-like plot of DAPC analysis on the dataset of *Triakis megalopterus*. Tme_SEAO and Tme_SWIO represents the South African South-East Atlantic and South-West Indian Ocean sampled populations, respectively. Each individual is represented by a vertical coloured line and each colour represents a genetic cluster (*k*).

4.4. Discussion

Recent advances in next-generation sequencing technologies have accelerated the mining of species-specific microsatellite loci in shark species generally devoid of molecular markers (Chabot and Nigenda 2011; Blower *et al.* 2015; Pirog *et al.* 2015). In this study, the use of Illumina HiSeq™ 2000 for reduced genome sequencing was successful regarding speed, accuracy, and cost in generating microsatellites. It provided an efficient way to develop microsatellite markers, even though some factors such as library preparation, read length and precision of the assembly can be improved. The relative richness of different types of microsatellite repeats is typical, and in sharks, dinucleotide repeats are generally over-represented. Similar to the studies of the Australian gummy shark *Mustelus antarticus* (Boomer and Stow 2010), the tope shark (Chabot and Nigenda 2011), and the brown smoothhound shark *M. henlei* (Chabot 2012) the current study found that dinucleotide microsatellite repeats were the most frequent repeat type present in the common smoothhound shark genome. Furthermore, two polymorphic multiplex assays were successfully constructed and optimised for the common smoothhound shark. The validation of the multiplex assays in the common smoothhound revealed similar genetic diversity indices as found in a previous study of the same species using cross-amplified loci (Maduna *et al*. 2016). Given that in sharks, microsatellite flanking sequences are conserved owing to low mutation rates (Martin *et al.* 2002), the cross-species amplification of orthologous microsatellite loci in other Triakidae species in the present study was assessed. A high cross-species amplification rate of success (> 70%) across all microsatellite loci was observed. Such findings were similar to those previously reported on sharks (Chabot and Nigenda 2011; Giresi *et al.* 2012; Maduna *et al*. 2014; Blower *et al.* 2015).

There is often a negative correlation between the evolutionary distance of the focal and target species, and the transferability of loci (amplification success and polymorphism) in sharks (Maduna *et al.* 2014). A similar trend has also been found in

several other vertebrate taxa including birds, amphibians and fish (Primmer *et al.* 2005; Carreras-Carbonell *et al.* 2007; Hendrix *et al.* 2010). All the species that were included in this study were closely related and accordingly the high performance of cross-species amplification was expected, albeit, the blackspotted smoothhound had the lowest transferability rate possibly due to the presence of null alleles. These loci, nevertheless, could prove useful in elucidating patterns of population genetic structure and gene flow within other Triakidae species. Besides the comparison of population genetic parameters among multiple closely related species, cross-species microsatellites can also be applied for species identification based on species-specific allele sizes at multiple loci, a technique that has rarely been used for forensic studies of sharks (Maduna *et al.* 2014; Marino *et al.* 2014; Giresi *et al.* 2015).

Assessment of the distribution of genetic diversity of the four co-distributed coastal sharks (the common smoothhound, spotted gully shark, tope shark and the whitespotted smoothhound) based on the newly developed multiplex assays indicated that the microsatellite loci are informative for species identification as well as for population genetic analysis. The preliminary population genetics estimates hinted at the combined effects of oceanographical barriers and life-history differences (*e.g.* mobility and sex-specific dispersal strategies) to be the major factors influencing the patterns of regional population structure in these sharks. The null hypothesis of panmixia was rejected in all the study species except for *T. megalopterus*. In line with previous studies by Bitalo *et al.* (2015) and Maduna *et al.* (2016) interoceanic genetic structure in the common smoothhound across the Atlantic/Indian Ocean boundary were detected. These findings also suggest the presence of fine-scale genetic structure in the whitespotted smoothhound, indicating that the unknown sampling population was collected along a gradient of restricted gene flow. Based on the Bayesian (STRUCTURE and GENECLASS) and multivariate (DAPC) analyses it is evident that most of the unknown samples came from the Atlantic Ocean. In *Mustelus* species, it seems intraspecific populations are typically connected via a series of stepping stone populations (Pereyra *et al.* 2010, Boomer 2013). In such systems, genetic structure is

usually reflected by a combination of effective population size, individual movements and migrations, seascape feature and habitat preferences *e.g.*, the narrownose smoothhound *M. schmitti* (Pereyra *et al*. 2010), the Australian gummy shark (Boomer 2013), the rig *M. lenticulatus* (Boomer 2013) and the brown smoothhound shark (Chabot *et al*. 2015, Sandoval-Castillo and Beheregaray 2015). Pereyra *et al*. (2010) and Boomer (2013) found no evidence of population genetic structure while Chabot *et al*. (2015) and Sandoval-Castillo and Beheregaray (2015) provided compelling evidence for the interplay of oceanography and dispersal differential between sexes in shaping genetic structure. In agreement with Maduna *et al.* (2016), the present study found asymmetric gene flow that predominantly occurs from the South-West Indian to South-East Atlantic Ocean for the common smoothhound, and a similar trend was observed for the whitespotted smoothhound. Granted, the reproductive and seasonal behaviour of the two study smoothhounds remains for the most part unknown (*sensu* Smale and Compagno 1997; Da Silva *et al*. 2013), particularly for the whitespotted smoothhound, however it appears that genetic structure in these species is highly similar (at least in the samples investigated here).

Results from previous research indicated that levels of gene flow across the Atlantic/Indian Ocean boundary for the tope shark was relatively high (Bitalo *et al.* 2015), yet in the present study significant interoceanic genetic structure with two genetic clusters characterised by lower levels of admixture (SEAO and SWIO) was found. The Bitalo *et al*. (2015) study, however, included only one Indian Ocean population (Struis Bay) near the proposed boundary and noted significant population differentiation between this SWIO sampling site and a SEAO sampling site, Robben Island. More recently, Bester-van der Merwe *et al*. (in press) also show support for an additional barrier besides the Atlantic/Indian Ocean boundary to influence genetic structuring of tope shark populations along the South African coast. In the latter study more sampling populations from east of the Atlantic/Indian Ocean boundary were included and agrees with what was found in the present study. Similarly, to smoothhounds, long-term gene flow estimates between ocean basins were

asymmetrical and mainly occurs from the South-West Indian to South-East Atlantic Ocean. The homogenous population structure observed here for the spotted gully shark was unexpected, given the available tagging data which indicate possible philopatric behaviour for the species, although, it freely travels across the Atlantic/Indian Ocean boundary (Dunlop and Mann, 2014; Soekoe 2016). However, it is well documented that the Atlantic/Indian Ocean boundary (Benguela Barrier) or transition zone is not fixed and extends from Cape Point (westernmost boundary) to Cape Agulhas (easternmost boundary) depending on the species in question (Teske *et al.* 2011). The former may hold true for the spotted gully shark given our sampling site that we used as a representative of the Atlantic Ocean (Cape Point and Betty's Bay).

Coalescent analyses for migration model comparison highly supported the model of the southward flux of migrants (i.e. migration from SWIO to SEAO) and showed that Θ was highest in the SWIO and lowest in the SEAO populations in all study species. The detection of similar asymmetric migration patterns in these species might suggest that such patterns arose from the action of shared physical boundaries. Also, water temperature changes have been shown to influence movement of these triakid sharks and other closely related species (West and Stevens 2001; Chabot and Allen 2009; Espinoza *et al.* 2011; Da Silva *et al.* 2013, Soekoe 2016). From the perspective of thermal physiology, albeit speculative, individuals from subtropical and/or warm-temperate bioregions can more easily colonise the cool-temperate bioregions as opposed to the reverse. Nevertheless, it is apparent that the cold Benguela Current and its interplay with the warm Agulhas Current also influence the patterns of gene flow in these coastal sharks as evident in a variety of other regional coastal fish species (Henriques *et al*. 2012, 2014, 2015) as well as passively dispersing marine species (Teske *et al.* 2015). Although population and genetic sampling in the present study are limited, the Agulhas Current presents a significant barrier to the northward migration in smaller coastal sharks.

4.5. Conclusions

In summary, this chapter reports on the validation of *in silico* predicted microsatellite loci for the common smoothhound shark and their potential applications in a biodiversity conservation context. The newly developed multiplex assays described in this study provided valuable molecular tools for species identification, assessing the distribution of genetic diversity and determining the directionality of gene flow, factors which are all vital for the conservation and management of these locally exploited shark species.

4.6. References

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4.7. Appendix

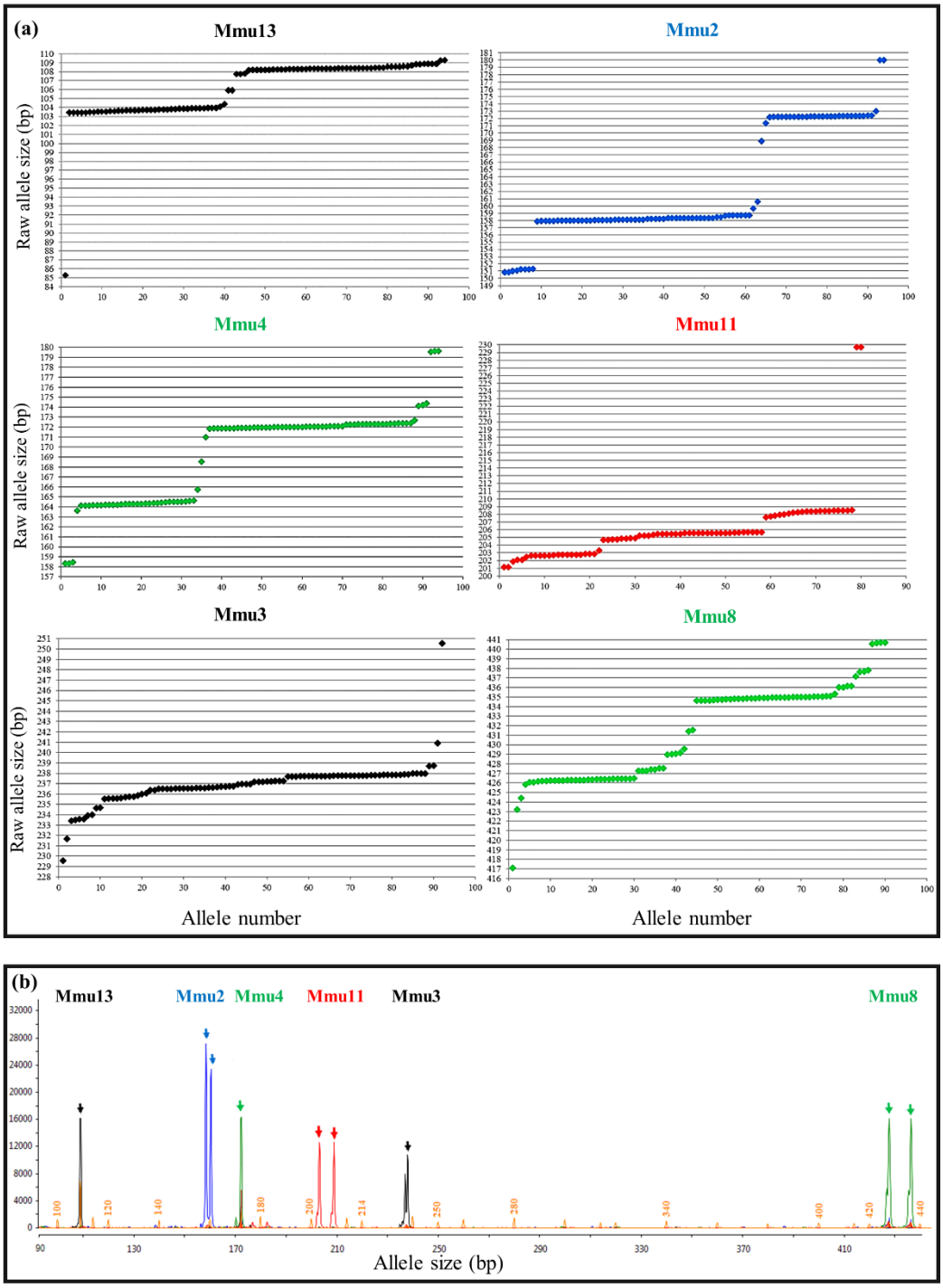

Figure A4.1 Binning and profiles for multiplex 1. (a) Allelograms based on 48 individuals from the South African South-East Atlantic and South-West Indian Ocean sampled populations, respectively. Here, the allele number corresponds to the ranking number of the allele in the list of allele raw sizes, ranked in increasing order. (b) Example of an individual electropherograms where arrows point to alleles at each locus, and small peaks with numbers (base pairs) correspond to fragments of the internal size standard LIZ600

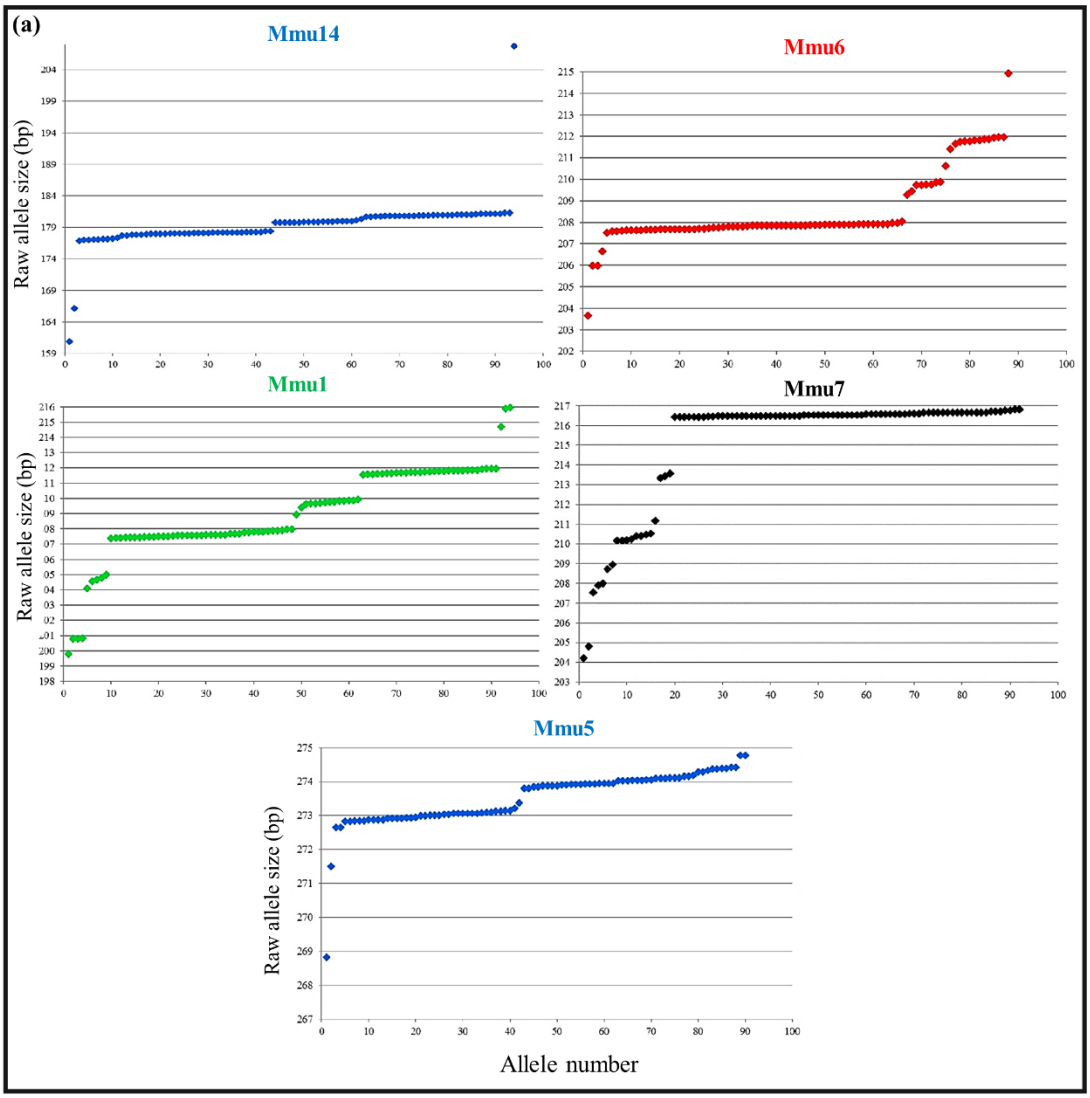

Figure A4.2 Binning and profiles for multiplex 2. (a) Allelograms based on 48 individuals from the South African South-East Atlantic and South-West Indian Ocean sampled populations, respectively. Here, the allele number corresponds to the ranking number of the allele in the list of allele raw sizes, ranked in increasing order. (b) Example of an individual electropherograms where arrows point to alleles at each locus, and small peaks with numbers (base pairs) correspond to fragments of the internal size standard LIZ600

Table A4.1 Characteristics of two polymorphic microsatellite multiplex assays for *Mustelus palumbes* based on two sampling ocean basins in South Africa, Southeast Atlantic Ocean (SEAO) and South-West Indian Ocean (SWIO). Primer concentration in the final reaction as μM/primer ([P]); Number of individuals (*N*), Number of alleles per locus (N_A); allelic richness (A_R); observed heterozygosity (H_O); expected heterozygosity (H_E); polymorphic information content (*PIC*); Probability of conformity to Hardy-Weinberg expectations (P_{HW}); null allele frequency (Fr_{NULL}). Mean values for each multiplex assay and overall are indicated in bold.

Locus	Repeat motif	[P]	Dye	Ocean basin	*N*	Size range (bp)	N_A	A_R	H_O	H_E	*PIC*	F_{IS}	P_{HW}	Fr_{NULL}
Mmu2	$(AC)_6$	0.2	FAM	SEAO	10	139-181	4	3.3	0.40	0.36	0.33	-0.108	1.000	0.000
				UKNOWN	15		3	2.3	0.14	0.20	0.19	0.307	0.111	0.000
				SWIO	12		9	6.9	0.67	0.85	0.79	0.225	0.002	0.038
Mmu3	$(TC)_7$	0.2	NED	SEAO	10	228-244	5	4.5	0.60	0.70	0.62	0.150	0.025	0.067
				UKNOWN	15		7	6.1	0.80	0.86	0.81	0.072	0.172	0.022
				SWIO	12		5	4.4	0.60	0.76	0.67	0.217	0.238	0.074
Mmu4	$(TG)_7$	0.2	VIC	SEAO	10	157-201	2	2.0	0.30	0.27	0.22	-0.125	1.000	0.000
				UKNOWN	15		6	4.1	0.80	0.65	0.57	-0.235	0.000	0.018
				SWIO	12		9	6.7	0.83	0.85	0.79	0.022	0.001	0.000
Mmu8	$(CAG)_5(TGT)_5$	0.3	VIC	SEAO	10	410-438	9	7.6	0.80	0.87	0.81	0.089	0.368	0.031
				UKNOWN	15		2	8.0	0.85	0.90	0.85	0.057	0.525	0.000
				SWIO	12		3	7.3	0.50	0.88	0.83	0.443	0.000	0.177
Mmu11	$(CAA)_5$	0.3	PET	SEAO	10	203-212	11	1.9	0.00	0.19	0.16	1.000	0.053	0.204
				UKNOWN	15		1	1.0	0.00	0.00	0.00	N.A.	N.A.	0.001
				SWIO	12		7	4.4	0.10	0.74	0.65	0.871	0.000	0.353
Mmu13	$(GCA)_5$	0.2	NED	SEAO	10	85-118	9	3.0	0.43	0.67	0.55	0.379	0.329	0.135
				UKNOWN	15		5	4.8	0.73	0.67	0.61	-0.096	0.203	0.026
				SWIO	12		7	5.3	0.50	0.71	0.65	0.309	0.014	0.057
MPS1 (mean)	**-**	**-**	**-**			**-**	**5.8**	**4.7**	**0.503**	**0.619**	**0.561**	**0.210**	**-**	**0.067**

Table A4.1 Continued.

Locus	Repeat motif	[P]	Dye	Ocean basin	*N*	Size range (bp)	N_A	A_R	H_O	H_E	*PIC*	F_{IS}	P_{HW}	Fr_{NULL}
Mmu1	$(AT)_7$	0.2	VIC	SEAO	10	180-214	2	2.0	0.20	0.53	0.38	0.633	0.075	0.200
				UKNOWN	15		6	4.8	0.73	0.71	0.65	-0.037	0.133	0.000
				SWIO	12		6	5.4	0.50	0.82	0.75	0.400	0.005	0.168
Mmu5	$(CTC)_6$	0.3	FAM	SEAO	10	253-291	3	2.9	0.30	0.62	0.49	0.526	0.061	0.199
				UKNOWN	15		6	4.5	0.54	0.66	0.60	0.196	0.159	0.012
				SWIO	12		7	5.3	0.58	0.77	0.70	0.252	0.001	0.103
Mmu6	$(CGC)_6$	0.3	PET	SEAO	10	203-213	4	4.0	0.50	0.76	0.67	0.357	0.034	0.124
				UKNOWN	15		6	5.0	0.64	0.73	0.67	0.127	0.611	0.018
				SWIO	12		6	5.7	0.56	0.81	0.74	0.328	0.012	0.129
Mmu7	$(GCT)_5$	0.2	NED	SEAO	10	201-219	4	3.5	0.56	0.58	0.48	0.036	1.000	0.000
				UKNOWN	15		6	4.7	0.64	0.62	0.57	-0.045	0.555	0.000
				SWIO	12		8	6.5	0.92	0.85	0.79	-0.080	0.071	0.000
Mmu14	$(AGC)_6$	0.2	FAM	SEAO	10	163-209	4	3.8	0.40	0.67	0.58	0.419	0.013	0.147
				UKNOWN	15		6	4.7	0.67	0.74	0.67	0.097	0.183	0.028
				SWIO	12		12	9.2	0.83	0.93	0.88	0.109	0.125	0.044
MPS2 (mean)	**-**	**-**	**-**			**-**	**5.7**	**4.8**	**0.571**	**0.720**	**0.641**	**0.221**	**-**	**0.078**
Overall (mean)	**-**	**-**	**-**			**-**	**5.8**	**4.7**	**0.534**	**0.665**	**0.597**	**0.215**	**-**	**0.072**

Table A4.2 Characteristics of two polymorphic microsatellite multiplex assays for *Galeorhinus galeus* based on two sampling ocean basins in South Africa, Southeast Atlantic Ocean (SEAO) and South-West Indian Ocean (SWIO). Primer concentration in the final reaction as μM/primer ([P]); Number of individuals (*N*), Number of alleles per locus (N_A); allelic richness (A_R); observed heterozygosity (H_O); expected heterozygosity (H_E); polymorphic information content (*PIC*); Probability of conformity to Hardy-Weinberg expectations (P_{HW}); null allele frequency (Fr_{NULL}). Mean values for each multiplex assay and overall are indicated in bold.

Locus	Repeat motif	[P]	Dye	Ocean basin	*N*	Size range (bp)	N_A	A_R	H_O	H_E	*PIC*	F_{IS}	P_{HW}	Fr_{NULL}
Mmu2	$(AC)_6$	0.2	FAM	SEAO	14	126-172	9	6.9	0.57	0.80	0.75	0.295	0.006	0.101
				UKNOWN	10		9	8.4	0.90	0.91	0.85	0.012	0.164	0.000
				SWIO	14	230-248	7	5.8	0.79	0.79	0.72	0.000	0.104	0.000
Mmu3	$(TC)_7$	0.2	NED	SEAO	10		5	5.0	1.00	0.80	0.72	-0.268	0.064	0.000
				SWIO	14	162-176	5	4.6	0.71	0.74	0.67	0.037	0.004	0.000
Mmu4	$(TG)_7$	0.2	VIC	SEAO	10		8	7.3	0.90	0.87	0.80	-0.038	0.081	0.000
				SWIO	11	405-436	10	8.5	0.82	0.90	0.84	0.095	0.007	0.000
Mmu8	$(CAG)_5(TGT)_5$	0.3	VIC	SEAO	8		9	9.0	1.00	0.93	0.85	-0.087	0.218	0.000
				SWIO	13	198-236	8	6.4	0.38	0.73	0.68	0.485	0.000	0.179
Mmu11	$(CAA)_5$	0.3	PET	SEAO	10		11	9.7	0.60	0.93	0.87	0.365	0.002	0.147
				SWIO	14	89-124	8	6.7	0.93	0.81	0.76	-0.154	0.394	0.000
Mmu13	$(GCA)_5$	0.2	NED	SEAO	10		6	5.2	0.70	0.57	0.52	-0.235	1.000	0.000
				SWIO		-	**7.9**	**7.0**	**0.78**	**0.81**	**0.75**	**0.042**		**0.036**
MPS1 (mean)	**-**	**-**	**-**			**-**	**5.8**	**4.7**	**0.503**	**0.619**	**0.561**	**0.210**	**-**	**0.067**

Table A4.2 Continued.

Locus	Repeat motif	[P]	Dye	Ocean basin	*N*	Size range (bp)	N_A	A_R	H_O	H_E	*PIC*	F_{IS}	P_{HW}	Fr_{NULL}
Mmu1	$(AT)_7$	0.2	VIC	SEAO	14	192-218	10	7.8	0.79	0.88	0.83	0.106	0.571	0.000
				SWIO	10		9	7.8	0.90	0.84	0.78	-0.073	0.045	0.000
Mmu5	$(CTC)_6$	0.3	FAM	SEAO	14	263-287	7	5.2	0.43	0.65	0.60	0.350	0.032	0.113
				SWIO	10		8	7.7	0.80	0.89	0.83	0.111	0.092	0.036
Mmu6	$(CGC)_6$	0.3	PET	SEAO	14	183-220	10	8.1	0.71	0.88	0.83	0.193	0.022	0.035
				SWIO	10		8	7.3	0.60	0.87	0.80	0.321	0.058	0.138
Mmu7	$(GCT)_5$	0.2	NED	SEAO	14	200-222	9	6.6	0.64	0.73	0.68	0.127	0.199	0.029
				SWIO	10		10	8.9	0.90	0.89	0.83	-0.013	0.088	0.000
Mmu14	$(AGC)_6$	0.2	FAM	SEAO	14	165-211	6	4.5	0.57	0.60	0.54	0.046	0.360	0.000
				SWIO	10		7	6.3	0.70	0.77	0.70	0.094	0.234	0.000
MPS2 (mean)	**-**	**-**	**-**			**-**	**8.4**	**7.0**	**0.70**	**0.80**	**0.74**	**0.126**		**0.035**
Overall (mean)	**-**	**-**	**-**			**-**	**8.14**	**7.00**	**0.74**	**0.81**	**0.75**	**0.080**		**0.035**

Table A4.3 Characteristics of two polymorphic microsatellite multiplex assays for *Triakis megalopterus* based on two sampling ocean basins in South Africa, Southeast Atlantic Ocean (SEAO) and South-West Indian Ocean (SWIO). Primer concentration in the final reaction as μM/primer ([P]); Number of individuals (*N*), Number of alleles per locus (N_A); allelic richness (A_R); observed heterozygosity (H_O); expected heterozygosity (H_E); polymorphic information content (*PIC*); Probability of conformity to Hardy-Weinberg expectations (P_{HW}); null allele frequency (Fr_{NULL}). Mean values for each multiplex assay and overall are indicated in bold.

Locus	Repeat motif	[P]	Dye	Ocean basin	*N*	Size range (bp)	N_A	A_R	H_O	H_E	*PIC*	F_{IS}	P_{HW}	Fr_{NULL}
Mmu2	$(AC)_6$	0.2	FAM	SEAO	16	153-181	8	7.7	0.94	0.71	0.65	-0.331	0.011	0.000
				UKNOWN	16		6	5.8	0.75	0.63	0.55	-0.192	0.001	0.023
				SWIO	16	230-250	4	3.8	0.19	0.18	0.17	-0.034	1.000	0.000
Mmu3	$(TC)_7$	0.2	NED	SEAO	16		4	3.9	0.19	0.24	0.22	0.211	0.065	0.000
				SWIO	16	164-180	4	3.9	0.19	0.29	0.27	0.357	0.075	0.114
Mmu4	$(TG)_7$	0.2	VIC	SEAO	16		4	3.9	0.19	0.29	0.27	0.357	0.075	0.114
				SWIO	16	412-426	2	2.0	0.06	0.42	0.32	0.854	0.002	0.257
Mmu8	$(CAG)_5(TGT)_5$	0.3	VIC	SEAO	16		4	3.9	0.13	0.34	0.31	0.636	0.001	0.146
				SWIO	15	148-238	7	7.0	0.40	0.59	0.54	0.328	0.042	0.123
Mmu11	$(CAA)_5$	0.3	PET	SEAO	15		4	4.0	0.40	0.44	0.39	0.102	0.077	0.073
				SWIO	16	100-116	3	2.9	0.75	0.51	0.40	-0.506	0.059	0.000
Mmu13	$(GCA)_5$	0.2	NED	SEAO	16		4	4.0	0.88	0.63	0.53	-0.414	0.133	0.000
				SWIO			**4.5**	**4.4**	**0.42**	**0.44**	**0.38**	**0.114**		**0.071**
MPS1 (mean)	**-**	**-**	**-**			**-**	**5.8**	**4.7**	**0.503**	**0.619**	**0.561**	**0.210**	**-**	**0.067**

Table A4.3 Continued.

Locus	Repeat motif	[P]	Dye	Ocean basin	*N*	Size range (bp)	N_A	A_R	H_O	H_E	*PIC*	F_{IS}	P_{HW}	Fr_{NULL}
Mmu1	$(AT)_7$	0.2	VIC	SEAO	16	202-216	3	3.0	0.69	0.64	0.54	-0.082	0.619	0.000
				SWIO	16		7	6.8	0.56	0.76	0.70	0.266	0.037	0.094
Mmu5	$(CTC)_6$	0.3	FAM	SEAO	16	271-283	4	3.9	0.13	0.59	0.49	0.794	0.000	0.278
				SWIO	16		4	3.9	0.38	0.59	0.49	0.373	0.102	0.111
Mmu6	$(CGC)_6$	0.3	PET	SEAO	16	204-218	6	5.9	0.69	0.68	0.62	-0.015	0.224	0.000
				SWIO	16		6	5.9	0.50	0.76	0.70	0.350	0.009	0.120
Mmu7	$(GCT)_5$	0.2	NED	SEAO	16	208-226	6	5.7	0.25	0.29	0.28	0.149	0.305	0.000
				SWIO	16		5	4.9	0.31	0.39	0.36	0.198	0.084	0.000
Mmu14	$(AGC)_6$	0.2	FAM	SEAO	16	177-214	5	4.8	0.25	0.24	0.22	-0.053	1.000	0.000
				SWIO	16		4	3.9	0.31	0.29	0.27	-0.087	1.000	0.000
MPS2 (mean)	**-**	**-**	**-**				**5**	**4.87**	**0.41**	**0.52**	**0.47**	**0.189**		**0.060**
Overall (mean)	**-**	**-**	**-**				**4.73**	**4.62**	**0.41**	**0.48**	**0.42**	**0.148**		**0.066**

Table A4.4 Analysis of molecular variance (AMOVA) for *Mustelus mustelus*, *Mustelus palumbes*, *Galeorhinus galeus* and *Triakis megalopterus*; *P <0.05, **P < 0.01.

Species	Source of variation	Variation (%)	F statistic	P value
Mustelus mustelus	Among populations	2.9	F_{ST} = 0.029	0.006**
	Within populations	-13.7	F_{IS} = -0.147	1.000
	Within individuals	110.8	F_{IT} = 0.108	1.000
Mustelus palumbes	Among populations	6.9	F_{ST} = 0.069	0.000**
	Within populations	17.5	F_{IS} = 0.188	0.000**
	Within individuals	75.6	F_{IT} = 0.244	0.000**
Galeorhinus galeus	Among populations	3.4	F_{ST} = 0.033	0.135
	Within populations	8.9	F_{IS} = 0.093	0.000**
	Within individuals	87.7	F_{IT} = 0.123	0.000**
Triakis megalopterus	Among populations	-1.2	F_{ST} = -0.012	1.000
	Within populations	13.6	F_{IS} = 0.134	0.000**
	Within individuals	87.6	F_{IT} = 0.123	0.000**

Table A4.5 MIGRATE-N model selection using the approximate log marginal likelihood (lmL) method. The Bézier approximation score was used to calculate the log-equivalent Bayes Factor (LBF) and select the most probable model (in bold) from among these four models. P_{Mi} is the model choice probability.

Model	No. of Parameters	Bézier lmL	LBF	P_{Mi}
Mustelus mustelus				
Full	4	-21557.47	2086.26	0.00
To SEAO only	**3**	**-20514.34**	**0.00**	**1.00**
To SWIO only	3	-20577.89	127.10	0.00
Panmictic	1	-29466.69	17904.70	0.00
Mustelus palumbes				
Full	4	-17365.13	5456.48	0.00
To SEAO only	**3**	**-14636.89**	**0.00**	**1.00**
To SWIO only	3	-14797.22	320.66	0.00
Panmictic	1	-21372.88	13471.98	0.00
Galeorhinus galeus				
Full	4	-12243.70	15635.98	0.00
To SEAO only	**3**	**-4425.71**	**0.00**	**1.00**
To SWIO only	3	-4502.19	152.96	0.00
Panmictic	1	-5765.09	2678.76	0.00

Table A4.5 Continued.

Model	No. of Parameters	Bézier lmL	LBF	P_{Mi}
Triakis megalopterus				
Full	4	-15757.39	12746.02	0.00
To SEAO only	**3**	**-9384.38**	**0.00**	**1.00**
To SWIO only	3	-9450.22	131.68	0.00
Panmictic	1	-12549.03	6329.30	0.00

Table A4.6 Results from MIGRATE-N for model 2 including parameters, the mode of the posterior distribution of the migration parameter *M* and bounds of 95% confidence intervals, the Θ and N_em (product of *M* and Θ divided by 4). SEAO is the South-East Atlantic Ocean and SWIO is the South-West Indian Ocean basins, respectively.

Species	Parameter	M mode	M 2.5%	M 97.5%	Mean
Mustelus mustelus	Θ_{SEAO}	0.79	0.40	1.32	0.86
	Θ_{SWIO}	5.87	4.94	6.88	5.91
	$M_{SWIO \rightarrow SEAO}$	37.95	29.00	49.70	38.63
	N_em	7.50			
Mustelus palumbes	Θ_{SEAO}	0.54	0.08	0.96	0.53
	Θ_{SWIO}	19.66	18.56	20.00	19.32
	$M_{SWIO \rightarrow SEAO}$	4.25	2.00	7.80	4.81
	N_em	0.57			
Galeorhinus galeus	Θ_{SEAO}	0.10	0.00	1.60	0.23
	Θ_{SWIO}	98.10	76.80	100.00	90.01
	$M_{SWIO \rightarrow SEAO}$	3.80	0.00	9.20	4.16
	N_em	0.10			
Triakis megalopterus	Θ_{SEAO}	1.38	0.28	15.36	4.77
	Θ_{SWIO}	6.82	5.64	8.04	6.85
	$M_{SWIO \rightarrow SEAO}$	89.40	52.00	146.40	96.59
	N_em	30.84			

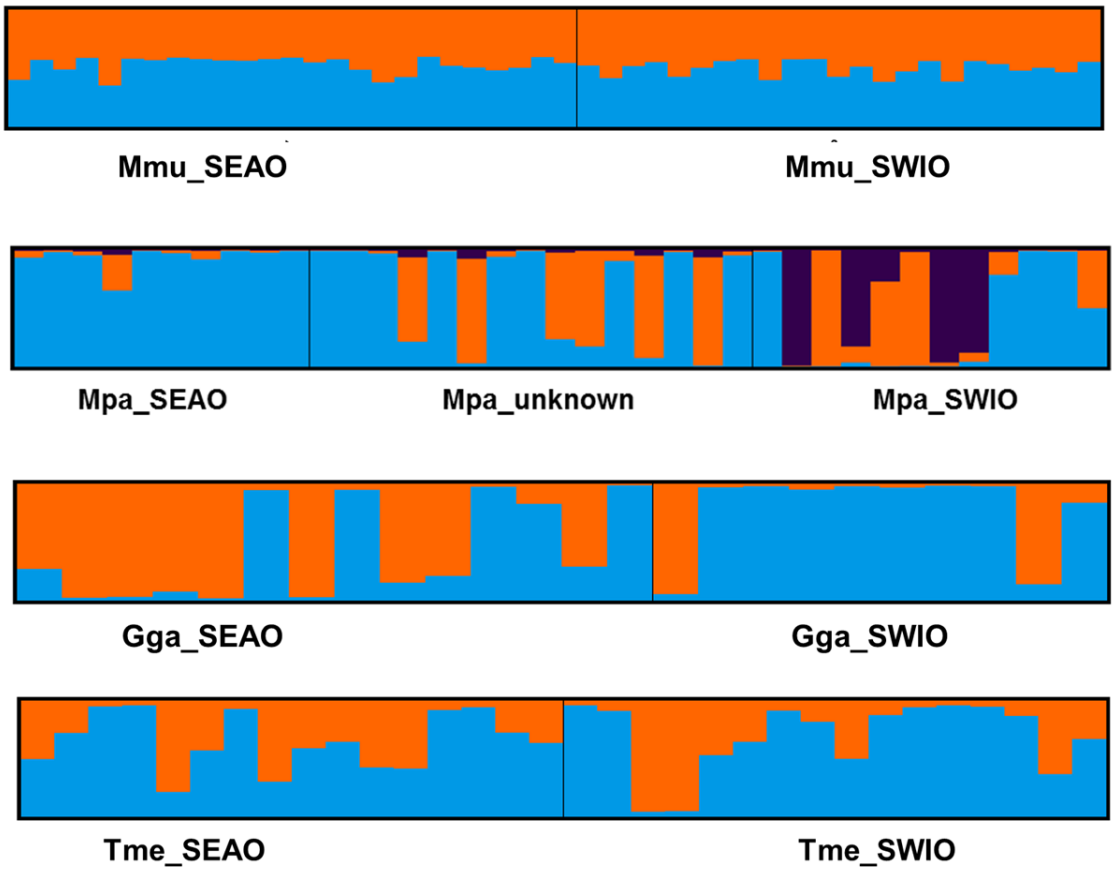

Figure A4.3 STRUCTURE results showing the most likely number of genetic clusters present in each of the four study species. SEAO and SWIO represents the South African South-East Atlantic and South-West Indian Ocean samples, respectively. Bar plots showing individual genotype membership to K clusters (each cluster is represented by a different colour, and each vertical bar represents an individual)

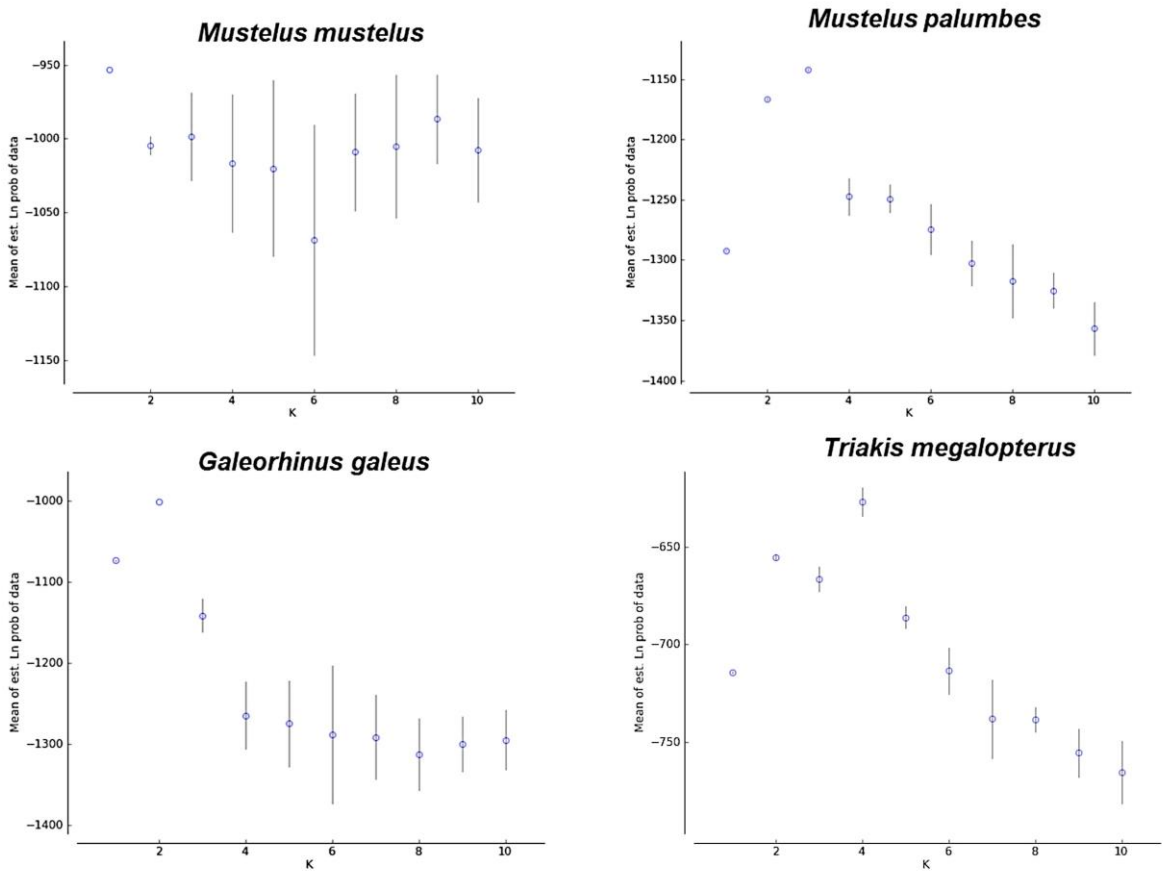

Figure A4.4 Likelihood probability profile estimated from STRUCTURE at K1-10 showing the mean and variance at each K for each study species.

CHAPTER 5

De novo discovery of SNPs in non-model species, the smoothhound sharks *Mustelus mustelus* and *M. palumbes*

Abstract

The value of single-nucleotide-polymorphism (SNP) research in conservation genetics is widely recognised and has barely commenced for non-model species such as sharks. Here, a *de novo* SNP discovery method, implemented in the bioinformatics tool DISCOSNP, was employed to identify SNPs in the common smoothhound shark (*Mustelus mustelus*). A subset of SNPs was selected for further validation using the Kompetitive Allele Specific PCR (KASP) SNP genotyping platform and for cross-amplication in the whitespotted smoothhound shark (*M. palumbes*). The SNP discovery pipeline described here enabled the identification of 26 826 putative SNPs of which 375 SNPs were retained for further analysis. For initial validation, a panel of 20 SNPs was selected and were optimised into simplex KASP assays. Cross-species amplification of the newly developed SNPs to the whitespotted smoothhound shark had a success rate of 100%. Although, for both species, after initial allele scoring only nine SNPs exhibited potential polymorphisms, the SNP discovery and genotyping approach used here will provide an alternative approach to marker development. In such a way, genome-wide data can be obtained in order to analyse population and demographic processes more accurately.

5.1. Introduction

The development of polymorphic—and hence informative—molecular markers is imperative for addressing fundamental population, ecological, evolutionary and conservation genetic questions for non-model species (Thomson *et al*. 2010; Dudgeon *et al*. 2012; Portnoy and Heist 2012). Non-model species, defined here as species lacking a reference genome, are increasingly investigated and the need for reference-free methods enabling the development of informative molecular markers has increased (Olsen *et al*. 2011; Helyar *et al*. 2011; Cruz *et al*. 2017). Next-generation sequencing (NGS) techniques have revolutionised molecular studies in non-model species, allowing for large-scale sequencing data to be generated at a reasonable cost (Everett *et al*. 2011; Helyar *et al*. 2011; Kumar and Kocour 2017). Generating enough sequence data for non-model species is no longer a limiting factor (Goetz *et al*. 2010; Künstner *et al*. 2010; Wolf *et al*. 2010; Helyar *et al*. 2011; Farrell *et al*. 2016; Maisano Delser *et al*. 2016), while on the other hand choosing a sequencing strategy or platform has become challenging (Davey *et al*. 2011; Ekblom and Galindo 2011; McCormack *et al*. 2013; Ellegren 2014; Kumar and Kocour 2017). Recent advances in NGS approaches have led to innovative cost-effective methods for discovering and genotyping of both microsatellite (Vartia *et al*. 2016; Farrell *et al*. 2016) and single nucleotide polymorphisms (SNPs) (Puritz *et al*. 2014; Campbell *et al*. 2015; Maisano Delser *et al*. 2016; Jiang *et al*. 2016) for non-model species. Most of these approaches allow for a reduced portion of the genome to be sequenced (*i.e*. reduced-representation library sequencing), resulting in multiple copies of the same DNA fragment obtained either from an individual sample or pools of samples to be compared (Narum *et al*. 2013; Willette *et al*. 2014). Moreover, NGS approaches are shifting towards the direct analyses of sequence variation, predominantly in the form of SNPs (Kumar and Kocour 2017).

A SNP is a nucleotide variant—or single DNA base substitution—found at a specific genomic location with a minor allele frequency (MAF) of at least one percent in a given population, characteristically bi-allelic with a lower mutation rate compared to length-based markers (Vignal *et al*. 2002; Brumfield *et al*. 2003; Morin *et al*. 2004). There is an increasing incentive to adopt SNPs as a marker of choice for studies on demographic and adaptive processes as well as fisheries forensics in non-model species (Morin *et al*. 2004; Williamson *et al*. 2007; Ramirez-Soriano and Nielsen 2009; Ogden 2011; Narum *et al*. 2013). This can be attributed to the many advantages SNPs hold over length-based markers (*e.g*. microsatellites) including high density throughout the nuclear genome (coding and non-coding regions), processing efficiency, ease in both scoring and standardising genotypes among laboratories (Brumfield *et al*. 2003; Morin *et al*. 2004; Garvin *et al*. 2010; Davey *et al*. 2011). Additionally, SNPs are assumed to evolve in a manner well described by a simple mutation models, such as the infinite sites model (Kimura 1969), upon which many population genetic approaches are based (Hedrick 1999; Meirmans and Hedrick 2010). Although the bi-allelic nature of individual SNPs results in a lower genetic power than that of individual microsatellites (Chakraborty *et al*. 1999; Krawczak 1999), SNPs can be genotyped with minimal error rate (Vignal *et al*. 2002; Helyar *et al*. 2011).

SNP discovery in non-model species can be divided into two main categories: *in vitro*-based methods and *in silico*-based methods. *In vitro* methods are based on the re-sequencing of targeted amplicons by utilising pre-existing DNA sequence datasets, such as expressed sequence tags (ESTs) libraries (Abadía-cardoso *et al*. 2011; Campbell and Narum 2011), or by creating new libraries (Campbell *et al*. 2009; Clemento *et al*. 2011). *In silico* methods on the other hand are based on the *de novo* screening of either restriction-site-associated (RAD) tags (Baird *et al*. 2008; Peterson *et al*. 2012; Davey *et al*. 2013) or sequencing of shot-gun libraries in target species (Cramer *et al*. 2008; Helyar *et al*. 2012; Cruz *et al*. 2017). For i*n silico* discovery, two reference-free methods can be used to detect SNPs: hybrid or *de novo* methods (Uricaru *et al*. 2015). Hybrid methods use both *de novo* assembly and mapping techniques to call SNPs (Willing *et*

al. 2011) while *de novo* methods, such as DISCOSNP, entails the direct identification of SNPs without having to assemble a full reference genome (Uricaru *et al*. 2015). Although *in silico* discovery provides an attractive approach for *de novo* SNP identification, it should be noted that with each approach there are advantages and disadvantages; and at present, there is no one ideal method for SNP discovery (Olsen *et al*. 2011; Puritz *et al*. 2014). Regardless of approach, a major challenge in SNP discovery is ascertainment bias, which is the systematic deviation from the expected allele frequency distribution—upward shift with an under-representation of rare SNPs—that occurs because of the unrepresentative sample of individuals that are used to discover or ascertain loci (Nielsen 2000; Wakeley *et al*. 2001; Brumfield *et al*. 2003; Bradbury *et al*. 2011). In theory, ascertainment bias is expected to influence inferences based on SNP allele frequency when the SNPs are identified from a limited number of samples but applied in a larger geographical context or *vice versa* (Clark *et al*. 2005; Albrechtsen *et al*. 2010; Bradbury *et al*. 2011). Several algorithms have been formulated to address and correct for ascertainment bias including the derivations of appropriate critical values and confidence intervals (*e.g*. Carlson *et al*. 2004; Voight *et al*. 2006) and directly correcting statistical estimators and statistics using specific models (*e.g*. Nielsen 2000; Wakeley *et al*. 2001; Nielsen and Signorovitch 2003; Marth *et al*. 2004; Nielsen *et al*. 2004).

Currently, there are an array of uniplex (single-plex) and multiplex SNP genotyping platforms that combine a variety of chemistries, detection methods, and reaction formats (Kumar and Kocour 2017). Kompetitive Allele Specific PCR (KASP™) is a single-plex SNP genotyping platform that uses a novel homogeneous fluorescent detection system (LGC Genomics, http://www.lgcgenomics.com). The KASP platform is based on allele-specific oligo extension and fluorescence resonance energy transfer (FRET) for signal generation allowing for bi-allelic scoring of SNPs or insertions and deletions (Indels) at specific loci (Kumpatla *et al*. 2012; Semagn *et al*. 2014). The genotyping cost for KASP depends on the number of data points (1 data point = 1 sample genotyped by 1 SNP) and data turnaround, which are 4 to 6 weeks

for normal turnaround and 2 to 3 weeks for rapid turnaround (LGC Genomics). The KASP platform allows for SNP genotyping at a user-defined number of polymorphisms, using DNA templates as short as 50 bp. The KASP platform has properties ideal for *de novo* SNP discovery in non-model species, such as low cost, low- to high-throughput analysis, accuracy, reproducibility, and flexibility, when compared with other marker systems (reviewed in Semagn *et al*. 2014).

In non-model species, such as sharks, the use of SNP markers is quite novel and has focused mostly on deciphering demographic and adaptive processes (Portnoy *et al*. 2015; Dimens 2016; Maisano Delser *et al*. 2016; Pazmiño *et al*. 2017), while more recently, also resolving molecular taxonomy issues (Corrigan *et al*. 2017). A few studies have employed SNPs to assess their power in addressing fundamental population, ecological and conservation genetics questions (Narum *et al*. 2008; Hess *et al*. 2011; Mesnick *et al*. 2011; Defaveri *et al*. 2013). For instance, Dimens (2016) found the exact same pattern of gene flow in the blacknose shark (*Carcharhinus acronotus*) using 2178 nuclear SNPs as appose to a previous study by Portnoy *et al*. (2014) that employed microsatellites and the mitochondrial control region. In the Corrigan *et al*. (2017) study, 2152 nuclear SNPs were able to distinguish between two superficially morphologically cryptic sharks, Galapagos sharks (*Carcharhinus galapagensis*) and dusky sharks (*Carcharhinus obscurus*), while the mitochondrial NADH2 marker failed to differentiate between these species.

The common and whitespotted smoothhound sharks are two demersal coastal species with economic and recreational value in southern Africa. These species are considered optimally exploited to overexploited in southern African waters. However, the misidentification of these sharks is a common occurrence and obscures the estimates of species-specific catch rates (Da Silva 2007; Da Silva and Bürgener 2007). To augment the existing molecular marker repository for the common smoothhound shark, the aim of the chapter is to (i) describe the *in-silico* discovery of SNP loci through *de novo* sequence clustering and contig assembly, (ii) validate a subset of *in-silico*-predicted SNP loci, and (iii) develop and optimise KASP genotyping

assays. Lastly, the chapter examines the cross-species transferability of the successfully validated SNP markers to a closely related species, the whitespotted smoothhound shark.

5.2. Materials and methods

5.2.1. Reduced-representation library construction and Illumina sequencing

A single finclip sample of the common smoothhound shark collected during sampling trips was used for SNP development. Total genomic deoxyribonucleic acid (DNA) was isolated using a modified cetyltrimethylammonium bromide extraction protocol of Sambrook and Russell (2001) (see Chapter 3) and sent to the Agricultural Research Council Biotechnology Platform in Pretoria, South Africa. One microgram of genomic DNA was used for 2 × 250 bp paired-end library preparation with a mean insert size of 400 bp using the Illumina TruSeq® DNA library preparation kit instead of the standard Illumina Nextera™ library preparation kit (Chapter 4). Sequencing of the library was performed on two lanes of an Illumina HiSeq™ 2000 sequencer per manufacturer's specifications. No inbred homozygous individuals of the common smoothhound shark have been reported (*sensu* Maduna *et al*. 2014, 2016; Marino *et al*. 2014, 2015, 2017; Chapter 4) and within-individual polymorphism was thus to be expected in the next-generation sequencing data generated in this chapter. A workflow illustrating the steps involved in processing of the data from the paired-end sequencing experiment is given in **Figure 5.1**.

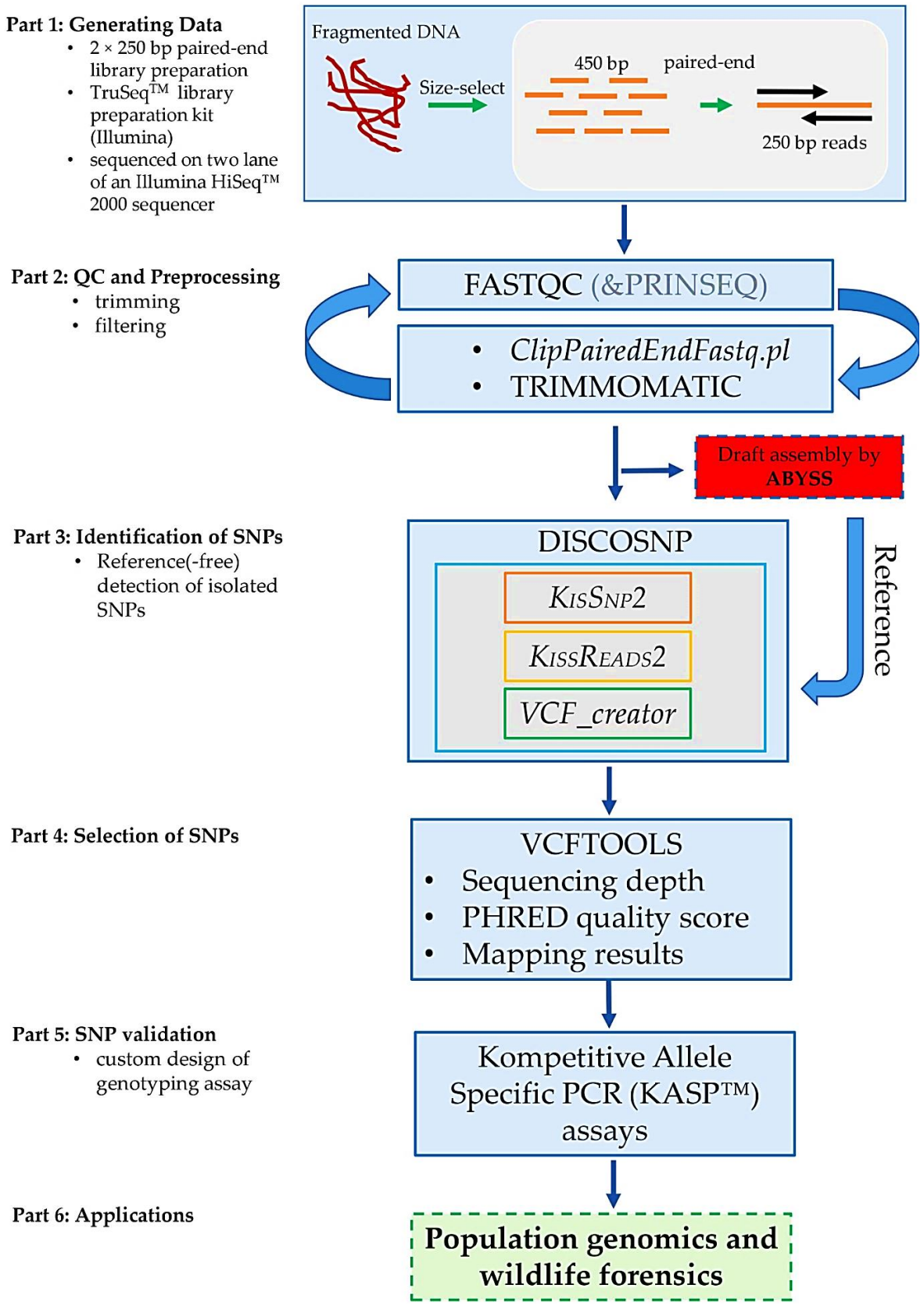

Figure 5.1 Schematic overview of genome assembly, SNP detection pipeline and applications (see text for more detail).

5.2.2. Sequence processing and de novo assembly

Raw sequencing reads were submitted to a quality control (QC) step to remove artificial duplicates and/or reads that contained any "Ns" using PRINSEQ v. 0.20.4 (Schmieder and Edwards 2011). Reads were quality-filtered and trimmed to remove all Illumina universal adapters as well as sequences shorter than 35 bp using TRIMMOMATIC v. 0.33 (Bolger *et al*. 2014) with default settings. A PHRED quality score of 15 was selected and filtered for sequences that contained at least 90% of the individual bases above this quality score.

A secondary QC step was performed in order to check whether primer, barcode, and adapter sequences have been properly trimmed by visualising the trimmed data in FASTQC v. 0.11.4 (Andrews 2010). In the present study, it was necessary to remove all adapter sequences using a perl-script *clipPairedEndFastq.pl* based on the CUTADAPT tools (Martin 2011) provided by ecSeq Bioinformatics (www.ecseq.com). After the QC steps, cleaned reads were used for sequence clustering first by merging the paired-end reads to produce a 'per contig' assembly using PEAR (Zhang *et al*. 2013), and then by assembly of the 'paired' FASTQ files using ABYSS v. 1.5.2 (Simpson *et al*. 2009). The consensus sequence produced for the contigs was then used as a draft reference genome for mapping reads in the subsequent *de novo* SNP discovery and *in silico* validation.

5.2.3. SNP detection and in silico *validation*

To identify candidate SNPs, the *de novo* method implemented in the program DISCOSNP v. 2.2.9 (composed of three independent modules, *KisSnp2*, *KissReads2* and *VCF_creator*) was used (Uricaru *et al*. 2015). Briefly, the *de novo* method is based on the observation that in the de Bruijn graph, a SNP generates a pair of paths (referred to as a bubble) composed of k vertices [*i.e*. the set of words of length k (k-mers) contained in the reads], which represent $2k$-1 length sequences that are polymorphic at one position. The first module, *KisSnp2*, constructs a de Bruijn graph by extracting all k

vertices from the reads and then detects bubbles to reveal the presence of a SNP in the read set(s). The second module, *KissReads2*, maps back input reads on the sequences of the predicted SNPs to remove spurious sequences not existing in reads and, to provide per allele coverage and per read set information (*i.e. in silico* SNP validation). The third module, *VCF_creator*, generates a VCF from *KisSnp2* or *KissReads2* with an option to use a reference file. In this study, DISCOSNP was run with default parameters, except for the -b (branch filtering strategy) option that was set to 1 (smart branching) in order to filter out SNPs for which two paths were branching. As a secondary validation step, a draft reference genome file was specified for *VCF_creator* using the -G option to provide a VCF file containing mapping results. The variant file was then filtered for bi-allelic SNPs that mapped to the draft reference genome (flagged 'PASS') using VCFTOOLS v. 0.1.14 (Danecek *et al*. 2011).

5.2.4. SNP assay development, validation and cross-species amplification

The uniplex SNP genotyping platform, Kompetitive Allele Specific PCR (KASP™) relies on the design of suitable allele-specific primers to enable bi-allelic scoring of SNPs (LGC Genomics). Here, a subset of 20 loci was selected based on the DISCOSNP rank score and flanking regions of at least 40 bp up- and down-stream of SNP position. Read alignments containing the selected SNPs were visualised in MEGA v. 7.0.14 (Kumar *et al*. 2016) and annotated using the Basic Local Alignment Search Tool (BLAST) and multiple sequence databases. BLASTn (query nucleotide against nucleotide database) searches, (E-value cut-off < 10^{-5}) were conducted against all annotated transcripts of five teleost fishes (fugu, medaka, stickleback and zebrafish) available through the Ensembl Genome Browser and the NCBI UniGene database. Also, BLASTx (translated query nucleotide against protein database) searches were conducted (E-value cut off < 1.0 E^{-3}) against the UniProtKB/SwissProt and UniProtKB/TrEMBL databases. For the KASP assay, two forward allele-specific primers differing at the terminal, 3′ nucleotide (which defines the SNP), and a common reverse primer were designed for each SNP loci using the web-based primer

allele-specific primer design tool WASP (Wangkumhang *et al*. 2007). In cases where no suitable primers could be identified with WASP, BATCHPRIMER3 (You *et al*. 2008) was used instead (**Table 5.1**). The specificity of the primers was then assessed *in silico* using PRIMER-BLAST (Ye *et al*. 2012).

For validation of the SNP assays, a diverse panel of 20 individuals of the common smoothhound was compiled by selecting a wide range of genotypes based on microsatellites previously reported for southern African populations (Maduna *et al*. 2016), and including additional samples collected from five sites across the species range in north-eastern Atlantic Ocean and Mediterranean Sea. All DNA extractions were carried out using the modified CTAB method (Sambrook and Russell 2001). The quality and quantity of DNA was verified using a NanoDrop ND 2000 spectrophotometer (Thermo Fisher Scientific; wwwthermofisher.com), after which all samples were standardised to 50 ng/µL. For KASP genotyping assays, the standardised DNA samples were sent to CenGen (Pty) Ltd, South Africa.

To summarise, the SNP-specific KASP assay mix and the universal KASP Master mix were added to DNA samples, a thermal cycling reaction was then performed, followed by an end-point fluorescent read. Bi-allelic discrimination was achieved through the competitive binding of two allele-specific forward primers, each with a unique tail sequence that corresponded with two universal FRET (fluorescence resonant energy transfer) cassettes, one of which was labelled with FAM™ dye and the other of which was labelled with HEX™ dye. Each assay was tested on the panel of 20 individuals, with a negative control and extraction blank per assay, in a 384-well plate. The KASP data was scored using KLUSTER CALLER v. 2.15 from LGC Genomics. Data displays were considered successful assays when two (or three, in the case of heterozygotes) distinct clusters with good separation were observed.

However, for several KASP assays there was ambiguity in discriminating the homozygous and heterozygous genotypes (see Results). Consequently, forward primers were designed and were used in combination with corresponding reverse KASP primers to allow for Sanger sequencing of the SNPs in order to establish

genotyping controls for each KASP assay. Polymerase chain reaction (PCR) was carried out on a GeneAmp® PCR System 2700 in a 10 µL reaction volume that included 50 ng of template DNA, 1x PCR Buffer, 200 µM of each dNTP, 0.2 µM of each primer, 1.5 mM $MgCl_2$ and 0.1 U of GoTaq® DNA polymerase. The PCR cycling conditions were as follows (i) one cycle of initial denaturation at 95°C for 2 min, (ii) 35 cycles of denaturation at 94°C for 30 s, optimized annealing temperature (T_A) for 30 s, elongation at 72°C for 2 min, (iii) a final elongation of one cycle at 60°C for 5 min and thereafter stored at 4°C. Optimum annealing temperature was determined by experimental standardisation for each of the primer pairs (**Table 5.2**).

Amplification products were sized using agarose gel electrophoresis and sequenced bi-directionally. Cycle sequencing was conducted in a total volume of 10 µL using the standard Sanger sequencing chemistry (BigDye® terminator v3.1 cycle sequencing kit; Life Technologies) and 1 pmol of each primer following the manufacturer's instructions. Capillary electrophoresis was conducted at the Central Analytical Facility, Stellenbosch University. Sequences were assembled into contigs (4 individuals, forward and reverse sequences), edited and aligned with the program MEGA. The SNP-containing contigs from the NGS data were used as references and homozygote and heterozygote genotypes were visually verified on the chromatograms.

To assess the utility of the newly developed SNP loci in related species, the whitesppoted smoothhound shark (*M. palumbes*) was genotyped for the full 20 KASP SNP panel.

5.3. Results

5.3.1. Sequence processing and de novo *assembly*

A total of 122 GB of raw reads were generated from the two sequencing runs of the common smoothhound shark reduced representation TruSeq® library. The removal of adapters was successfully performed using TRIMMOMATIC, however,

adapter sequences were still evident from the FASTQC output files (**Figure 5c** and **5d**). A secondary adapter removal step was conducted with the aid of a custom perl-script for trimming adapter sequences of paired-end experiments (**Figure 5e** and **5f**). After trimming the raw sequences that included removal of adapters, N-containing reads, and low-quality reads, approximately 60 GB of clean reads were retained. A total of 832 151 contigs with an average length of 210 bp were recovered following the *de novo* assembly of the Illumina paired-end reads.

5.3.2. SNP detection and selection of candidate SNPs for KASP assays

De novo SNP discovery with DISCOSNP involved estimating the main parameter, *k* value, by plotting the *k*-mer counting histograms for distinct *k* values. The best *k* value was determined to be $k = 31$ with a minimal coverage of 4. DISCOSNP detected 26 826 putative SNPs from the quality trimmed and filtered read files. Of the 26 826 putative SNPs, 9 185 SNPs (34 %) mapped onto the 'reference genome'. From the mapped SNPs, 3263 were located in contigs larger than 100 bp while only 475 of these SNPs had the required minimum of 50 bp flanking region to design primers for the KASP assays. In the present study, the rank score was not well suited to select SNPs that are heterozygous since ranking favours SNPs for which one variant is enriched in one read set, and therefore more suitable for a dataset of more than one diploid individual (Uricaru *et al*. 2015). Instead, the mapped SNPs were sorted according to their sequencing depth and PHRED quality score. Only those SNPs for which the sequencing depth was between 4 and 10, and a PHRED sequence quality of 20 were retained. As a result, only 375 SNPs remained. From those 375 SNPs, 20 SNPs were selected at random without taking BLAST hits into account (**Table 5.1**).

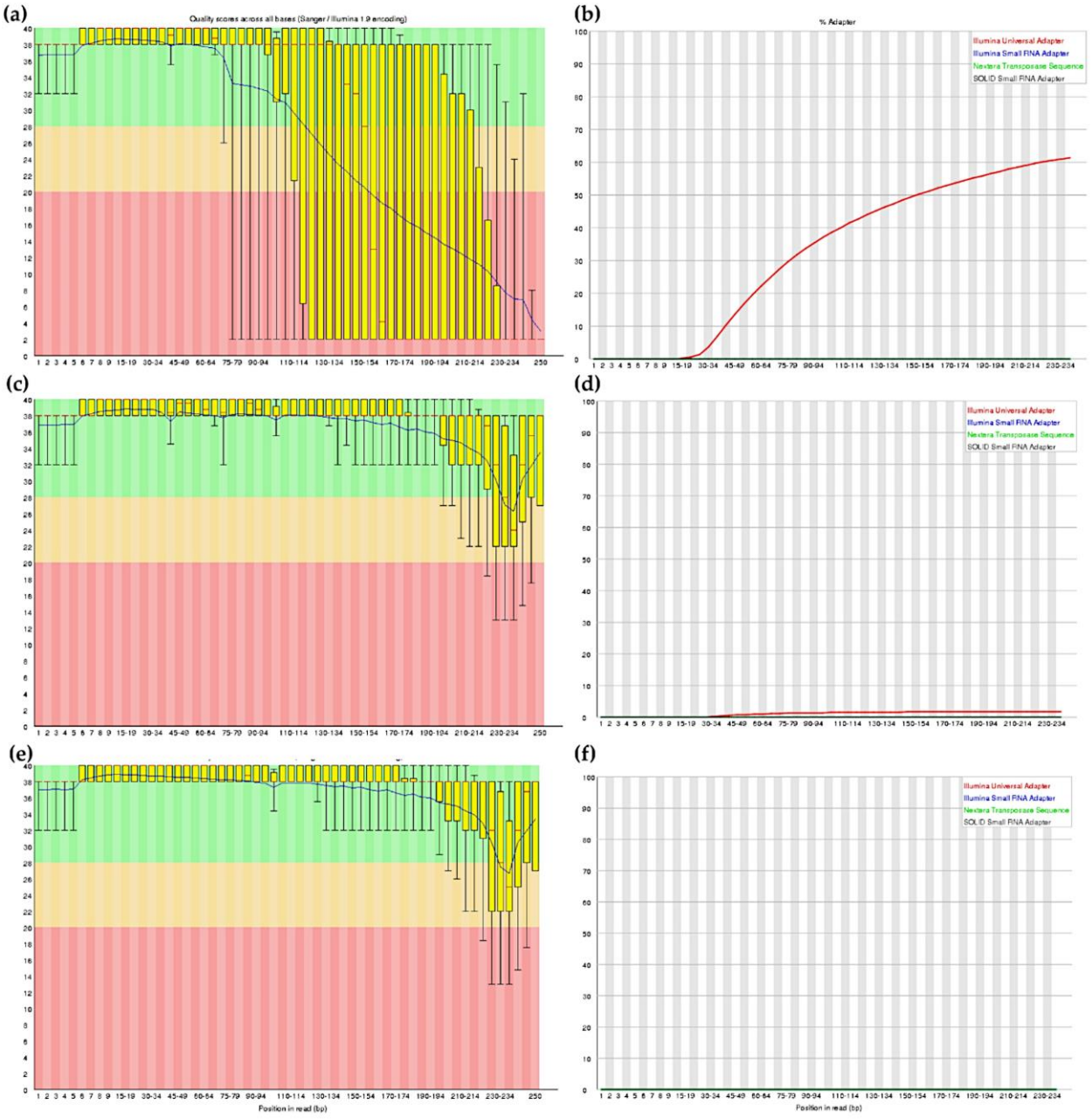

Figure 5.2 Sequence processing to remove adapter sequences from individual FASTQ files. a) sequence quality and b) adapter content for the raw data; c) base sequence quality and d) adapter content for the TRIMMOMATIC trimmed data; e) per base sequence quality and f) adapter content for the trimmed data using the perl-script *clipPairedEndFastq.pl*, respectively.

Table 5.1. *Mustelus mustelus* SNP primers used in the KASP SNP genotyping assays, expected product sizes and annotation. Allele-specific primers differ at the 3′ terminal nucleotide (in bold) and each includes a distinctive sequence of nucleotides at the 5′ end that are complementary to separate universal FRET cassettes contained in the KASP reagent (not shown). The reverse common primer is used during amplification of products containing both SNPs in the KASP assay.

SNP ID	Forward allele-specific primers (5′-3′)[a]	Reverse common primer (5′-3′)[b]	Product size (bp)	Annotation
MmuSNP002540	CATGTCCTCGGTGTCAATG(**C/G**)	AGCAGTGACTTGATTGAAGCCT	52	No homology
MmuSNP003636	GCTCCATCGCTCCCTCA(**A/G**)	GTCCTCCATCCAAACCACAG	130	No homology
MmuSNP005024	GCTTTGCAATGCCGTTT(**C/T**)	TAAGGGAGCTCCGACTCAAA	104	No homology
MmuSNP042364	ACATTTGCAATGCTCGG(**C/T**)	GCAGCTCCCATATTATCCCA	95	No homology
MmuSNP100616	ACTTAACGTCTTCATAGTTGCATT(**A/G**)	ATGTTCACTCCAATGCCTG	197	No homology
MmuSNP118808	AGTGTTCATGTTCACACACACCTA(**A/C**)	GCAGGAAGTATTGAAGGCGA	119	No homology
MmuSNP146579	CCAACAGCTAGTATCACCACAGA(**C/T**)	GTCCCGGTGTAACTGGACTA	54	No homology
MmuSNP171517	CTCCCTTCAGCTCTGTCG(**C/T**)	GCCAATGGGATTGCTCTG	70	No homology
MmuSNP268689	GTTCCATGGTTTCTCCATC(**A/G**)	CAAGGCTTGAACAGGTCATTT	69	No homology
MmuSNP274931	GGCCCAACCATCTTTC(**A/G**)	TCGTGAATGGTGCTGAACAT	105	No homology
MmuSNP280121	TTGTGGATTTGAAATTGACTC(**C/T**)	ACATTGGTTGTCTTGCTGTT	169	No homology
MmuSNP295381	CACGTCCATTGCGACCA(**A/G**)	GCCGCCATCTTGTTTCAG	68	No homology
MmuSNP313786	CCTCAGTGTAGCGGAGAAAC(**A/G**)	AGACCTGGTGGTGAAGCTGA	84	No homology
MmuSNP343457	AAAGCCGTGGACTGCAA(**C/G**)	CCAAATTCGTTCTCTCTCAC	99	No homology
MmuSNP362042	TCATTAATATAATGGGTAACACCTC(**A/G**)	TACCATCCTCAAAGTTCGGG	121	No homology
MmuSNP368851	CTGCCAATTAGGAGTGACC(**A/G**)	CACTCCTAATTGACAGCGGG	96	No homology

Table 5.1. Continued.

SNP ID	Forward allele-specific primers (5′-3′)	Reverse common primer (5′-3′)	Product size (bp)	Annotation
MmuSNP392347	TGAAAGCTTCAAGAACAGTCG(**C/G**)	AAATGGCAATCGAAGTGAATG	68	No homology
MmuSNP404837	GGAAATCATTGCTGGAGTTCTT(**T/G**)	AAAGACTTGGCGTTCTCTCG	68	No homology
MmuSNP411135	CATATACCGTGCCCCTCAC(**C/T**)	AAATGTCCTCGGATACTGCG	53	No homology
MmuSNP413058	ATGCACCCATGGTTAGTGTAA(**A/G**)	CCTAAGGACGACCCTCACT	54	No homology

Table 5.2. Variants and SNP primers, expected product sizes and annealing temperature (T_A) of nine SNPs in *Mustelus mustelus* with potential polymorphisms based on the KASP assays initial SNP calls. *The reverse primers used here were obtained from the KASP assays.

SNP ID	Variant	Forward primer (5′-3′)	Reverse primer (5′-3′)*	Product size (bp)	T_A (°C)
MmuSNP100616	A/G	GTGTCCCAGGCCCAAGTATT	ATGTTCACTCCAATGCCTG	289	59
MmuSNP146579	C/T	ACTCCAGAACAAGCAGGGAA	GTCCCGGTGTAACTGGACTA	198	58
MmuSNP274931	A/G	CTGAATGTGGTATTAAGGAGCCC	TTCAGGACAAGTGGGGATGG	273	58
MmuSNP295381	A/G	AGAGGTTCTGAACCAGCC	GCCGCCATCTTGTTTCAG	94	59
MmuSNP368851	A/G	CTGTCAATTAGGATTGAAC	CACTCCTAATTGACAGCGGG	116	58
MmuSNP392347	C/G	ACGGAGCCAGTTTGATGCTA	AAATGGCAATCGAAGTGAATG	130	59
MmuSNP404837	T/G	ATATGCATTTGGGAAAAGGATGC	AAAGACTTGGCGTTCTCTCG	100	59
MmuSNP411135	C/T	GTTGCAGACGGAGAGCGG	AAATGTCCTCGGATACTGCG	181	59
MmuSNP413058	A/G	TTCAGGACAAGTGGGGATGG	CCTAAGGACGACCCTCACT	205	59

5.3.3. SNP validation and cross-species amplification

In this study, two no-template controls (NTCs) were used and no false-positive signals were detected since the NTCs had either no signal or very weak signal (**Figure 5.3**). In other words, the NTCs did not produce signals exceeding the threshold values for autoscoring with the KLUSTER CALLER allele-calling software. However, for several KASP assays with a higher rate of uncalled SNPs the NTCs clustered together and overlapped with the unassigned genotypes due to poor amplification or failure to amplify (**Figure 5.3**). From the initial 20 SNPs that were genotyped, 11 were excluded either due to failure to amplify, large missing data points and/or ambiguity in discriminating the homozygous and heterogeneous genotypes on the genotype plots. Further analyses were conducted using the remaining nine KASP SNP assays which also showed promise to be polymorphic (**Figure 5.3**). The call rate for the nine SNPs ranged from 36.4% to 100%, where the lowest rate was due to unassigned genotypes.

To minimise the unassigned calls, forward common primers for each of the nine SNPs were designed to enable Sanger sequencing of amplicons to establish positive genotyping controls for each SNP variant. The KASP assay can be rescored in the presence of the recognised genotype controls for the individual SNP variants so to increase the SNP call rates.

To assess the cross-species utility of the panel of SNPs developed for the common smoothhound shark, these assays were also tested on the sympatric and closely related species, the whitespotted smoothhound shark. Cross-species amplification rate of success was 100%, although for a majority of the SNPs, the individuals of the whitespotted smoothhound shark scored homozygous for the alternative allele *e.g.*, in all KASP assays except for SNP146579 (Assay#007) and SNP392347 (Assay#017) shown in **Figure 5.3**.

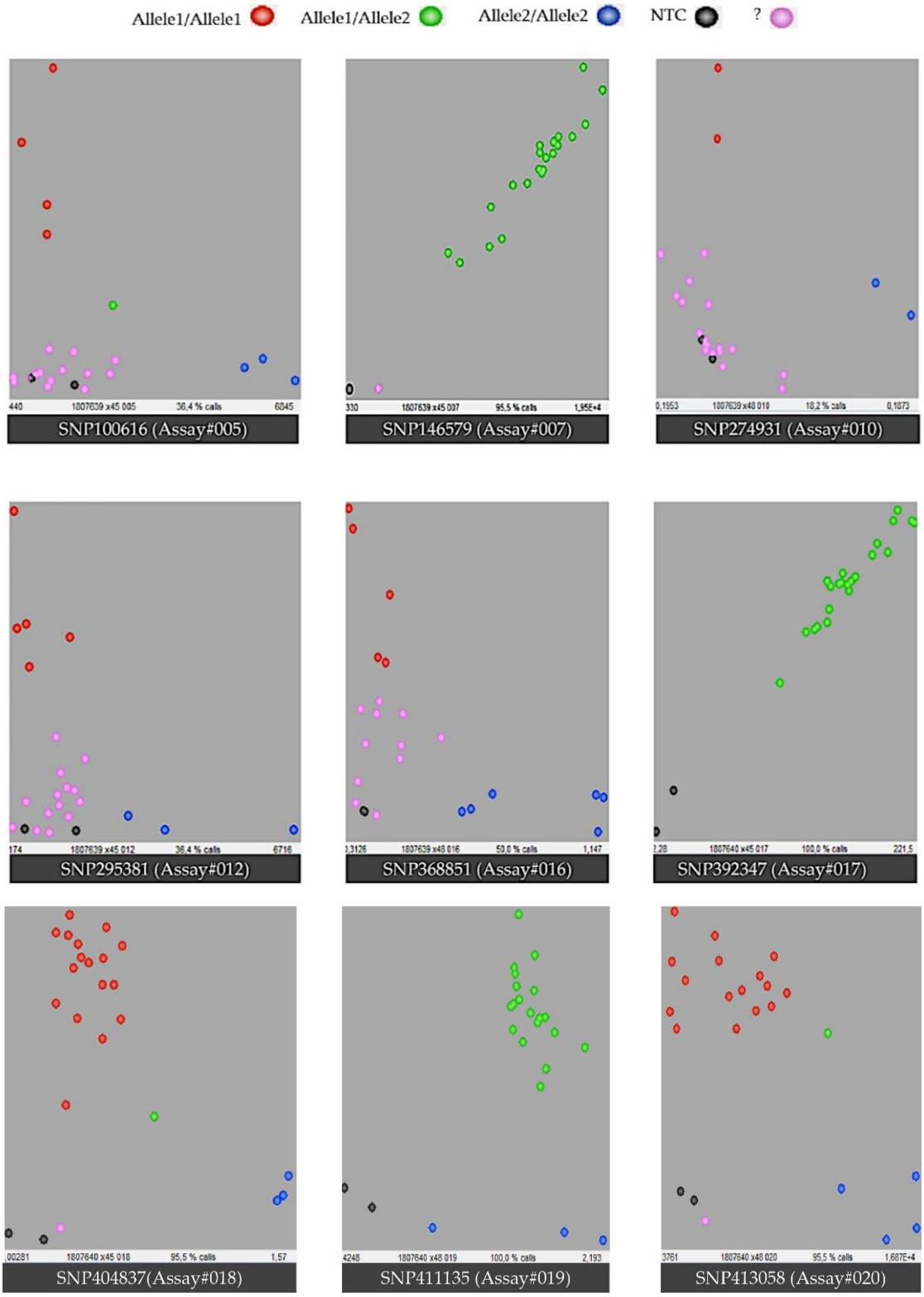

Figure 5.3 Genotype plots for the nine KASP assays for single nucleotide polymorphism (SNPs) identified in the genome of *Mustelus mustelus*. Homozygotes for allele 1 (red), heterozygotes with the alternative SNP alleles, homozygotes for allele 2 (blue), no-template control (NTCs, black) and genotyping failures (pink) are indicated. FAM and HEX fluorescence units are shown on the *x*- and *y*-axes, respectively. Assay (#) number is the number of the assay from the panel of 20 SNPs.

5.4. Discussion

Many species of smoothhounds (genus *Mustelus*) are of conservation and management concern. Development of genetic tools such as genome-wide markers in these sharks has been hampered in part by the lack of a reference genome of any closely related species. Cross-species utility of genetic tools has allowed for rapid marker discovery in wild relatives of taxa with well-developed resources. For instance, microsatellites in sharks with no genetic information were previously obtained through cross-species amplification from closely related species (Boomer and Stow 2010; Maduna *et al*. 2014; Pirog *et al.* 2015).

The use of cross-amplified microsatellites does however receive some dissent from population geneticists due to the chance of coincidentally amplifying non-orthologous microsatellite loci (for a thorough discussion see Chapter 4 and Maduna *et al*. 2014). The continued use of cross-amplified microsatellites for elasmobranch studies probably stems from the lower rates of molecular evolution generally observed in sharks (Martin *et al.* 2002). The increase in *de novo* microsatellite development in sharks can be attributed to the availability of next-generation sequencing (NGS) methods such as pyrosequencing in recent years (Boomer and Stow 2010; Chabot 2012; Taguchi *et al*. 2013; Pirog *et al*. 2015).

Developments in DNA sequencing technologies over the past decade have also enabled the rapid and extensive genome-wide investigations of sequence variation including SNPs (Kumar and Kocour 2017). The lack of established reference genomes in non-model species mandatory for the development of molecular markers is now circumvented using sequencing approaches e.g., reduced representation sequencing approaches. These approaches include genotyping-by-sequencing (GBS), restriction site-associated (RAD) sequencing (Helyar *et al*. 2011; Narum *et al*. 2013; Andrews *et al*. 2016) and transcriptome sequencing (RNA-seq) (De Wit *et al*. 2012). These approaches are all increasingly being employed for SNP discovery and genotyping in sharks (Portnoy *et al*. 2015; Dimens 2016; Pazmiño *et al*. 2017). Recently, such an effort in the

Galapagos shark (*Carcharhinus galapagensis*) produced a total of 8103 nuclear SNPs that were used in assessment of neutral and adaptive genetic diversity (Pazmiño *et al*. 2017).

The present chapter used Illumina HiSeq 2000 sequencing technology to augment an existing draft common smoothhound shark genome assembly also generated from Illumina sequence data of the same individual (see Chapter 4). The present chapter, however, aimed to use the sequence data to determine the feasibility of producing a new set of genetic markers *i.e*., SNPs, for the species and assess their cross-species utility in a close relative, the whitespotted smoothhound shark. This study is, to the author's knowledge, the first to report SNP discovery, provide optimised KASP SNP assays and report on the transferability of SNPs for any *Mustelus* species.

The SNP discovery pipeline described here enabled the identification of 26 826 putative SNPs (of which 375 SNPs were retained) in the common smoothhound shark genome. A possible reason why only 34% of SNPs mapped to the common smoothhound shark draft genome, is that two different library preparation methods were used while sequence data had been filtered out during the initial assembly of the draft genome. Regardless, the retained data also allowed for the successful design of primers for SNP genotyping using the KASP approach and nine optimised KASP assays. The traditional Sanger sequencing strategy was successfully employed to validate the putative SNPs that showed promise to be informative. Initial allele scoring on the KLUSTER CALLER software indicated that positive control DNA samples of known genotypes were required for reliable SNP calls. The Sanger approach then also served as the means for identifying the alternative homozygous and heterogeneous genotypes that could maximise genotype calls during allele scoring on the KLUSTER CALLER software. Although the KASP SNPs should still be re-scored using genotyping controls, it is evident that the KASP methodology will aid the rapid and cost-effective generation of genotypic data in sharks. Further analyses of the SNP data set generated in this chapter will allow for a larger panel of KASP SNP assays to be optimised.

Once sufficient KASP genotypic data has been generated, the magnitude of ascertainment bias arising from the nature of the unrepresentative discovery panel used in this chapter can also be assessed. This would involve genotyping multiple populations across the species distribution range to allow allele frequency spectrums of individual populations to be analysed. An allele frequency spectrum is the distribution of allele frequencies (minor allele frequencies, MAF) of polymorphic sites, and is reflective of the demographic and molecular processes experienced by a population (Ewens 1972; Marth *et al*. 2004; Nielsen *et al*. 2004). This distribution is also sensitive to ascertainment bias (Nielsen 2000; Wakeley *et al*. 2001) and empirical evidence demonstrates that an un-biased SNP panel should exhibit an ''L-shape'' distribution of MAF categories indicating adequate representation of low MAF SNPs (Marth *et al*. 2004; Nielsen *et al*. 2004).

The predicted SNPs reported in this chapter further provide the foundation for exploring alternative SNP genotyping methods such as genotyping-by-sequencing (GBS). Unlike the low marker density KASP genotyping platforms, GBS is a high marker density genotyping approach that adapts NGS protocols (*e.g*., target enrichment or reduction of genome complexity) to simultaneously discover and score segregating markers such as SNPs in populations of interest (Davey *et al*. 2011; Narum *et al*. 2013). To date, comparative surveys on the correlation between the KASP-based SNP assays and the GBS-based high density SNPs show only numerical differences, while the overall conclusions drawn from both methods are mostly similar (Ertiro *et al*. 2015).

The cross-species transferability of the novel SNPs for the common smoothhound shark to the whitespotted smoothhound indicated the potential use of these SNPs in other species of smoothhounds for population genetics studies and/or species identification purposes. Miller *et al*. (2010) showed that SNP assays developed for one species (source) were not likely to be useful in others (target) when the authors tested the OvineSNP50 BeadChip, developed for domestic sheep (*Ovis aries*), in two related ungulates (Bovine and Equine). In the former study, the cross-species call rate

decreases approximately 1.5% with each million-year divergence between the source- and target species, while retention of polymorphisms showed an exponential decay. On the other hand, Ogden *et al*. (2012) tested the BovineSNP50 BeadChip, developed for cattle (*Bos taurus*), in two antelope species, the scimitar-horned oryx (*Oryx dammah*) and Arabian oryx (*O. leucoryx*). Of the 54 001 SNP markers on the array, Ogden *et al*. (2012) found 148 polymorphic markers in the scimitar-horned oryx and 149 in the Arabian oryx, even though previous phylogenetic inferences suggested a divergence time of 23 million years between *Bos* and *Oryx* genera (Matthee and Davies 2001). A relatively higher success-rate is anticipated for congeneric shark species given that all known populations are from the wild and not inbred domestic populations such as in the case of the sheep and cattle breeds.

5.5. Conclusions

The results presented in this chapter provide a framework for a SNP discovery approach, *de novo* SNP identification and KASP SNP genotyping technology to be applied in non-model shark species in future. This approach could provide increased availability of genomic information for fine-scale resolution of population structure and phylogeography as well as wildlife forensics in the smoothhound shark genus *Mustelus*. Since the usefulness of the newly developed SNP panel remains untested, the current chapter should be viewed as a stepping stone for SNP discovery and application in smoothhound sharks. Further SNP panel selections and genotyping of more populations to assess the full extent of ascertainment bias is necessary and currently underway.

5.6. References

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CHAPTER 6

Testing molecular forensic methods to assist with species identification of southern African hound sharks

Abstract

Houndsharks (Carcharhiniformes: Triakidae) represent a substantial portion of shark catch in the southern Africa demersal fisheries. Misidentification of houndsharks is a prominent issue in fisheries due to the extensive overlap in external morphology among species. Since reliable catch data are essential for effective fisheries management, it is prudent that species are identified to the species level in order to account for the differences in species susceptibility and population vulnerability to exploitation. The aim of the study was to develop morphological and molecular screening tools for the identification of six houndsharks (*Galeorhinus galeus*, *Mustelus mosis*, *M. mustelus*, *M. palumbes*, *Scylliogaleus quecketti* and *Triakis megalopterus*). Two species diagnostic morphometric measurements, which include the dorsal origin to caudal tip length (L_{DOCT}) and dorsal origin to precaudal tip length (L_{DOPCT}) were used here for the two commercially important species of *Mustelus*, the common smoothhound (*M. mustelus*) and whitespotted smoothhound (*M. palumbes*). Examination of dermal denticle morphology revealed the presence of species-specific diagnostic characters such as shape and distribution. Two molecular assays were successfully developed for species identification of six houndsharks, including the use of a microsatellite panel and HRM analysis. This could complement traditional morphological keys such as the dressed morphological key and enhance the collection of species-level data (annual landings, catch rate, and bycatch/discard level) required for basic resource assessments.

6.1. Introduction

The commercial exploitation of sharks is a global occurrence and is primarily driven by the demand of shark related products including meat, fins, skin, cartilage, liver and teeth (Dulvy *et al*. 2014, 2017). There is increasing awareness of the vulnerability of sharks to overexploitation due to their *k*-selected life-history characteristics, which include slow growth, late maturation, long reproductive cycles and life spans, and low fecundity (Cortés 2000; Frisk *et al*. 2001; Dulvy *et al*. 2014). For those few shark species for which longer-term catch data are available, there is an indication of population declines attributed to overfishing and incidental capture (Musick *et al*. 2000; Baum *et al*. 2003, 2004; Dulvy *et al*. 2008). Although mounting evidence suggest that population declines stems from the lack of historical fisheries management measures, a current survey shows that at least 9% of the current global catch of sharks are biologically sustainable (Simpfendorfer and Dulvy 2017). Nonetheless, for most commercially important shark species, species-level data (annual landings, catch rate, and bycatch/discard level) required for basic resource assessments are lacking (Baum *et al*. 2003; Lack and Sant 2009; FAO 2014). This is especially pronounced in multi-species fisheries where obtaining data on shark catch and trade for individual species are complicated by species identification issues (Pank *et al*. 2001; Da Silva and Bürgener 2007; Mendonça *et al*. 2010; Marino *et al*. 2017). Identifying sharks during port inspections is extremely difficult (or even impossible) when using traditional taxonomic tools because of carcass processing at sea (Stevens 2004; Abercrombie *et al*. 2005; Akhilesh *et al*. 2014). During processing, heads and fins are removed, and morphological and meristic criteria used for identification of specimens are lost (Da Silva and Bürgener 2007; Mendonça *et al*. 2010). This has forced fisheries management to be based on aggregate species groups instead of individual species (Da Silva *et al*. 2015; Giresi *et al*. 2015; Marino *et al*. 2017). For effective fisheries management, it is prudent that species are identified to the species level in order to

account for the differences in species susceptibility and population vulnerability to exploitation.

Molecular–based methods for shark species identification have long been promoted as alternatives to resolve identification issues (Ward *et al*. 2008, Blanco *et al*. 2008, Naylor *et al*. 2012) or reveal captures of threatened shark species (Shivji *et al*. 2005, Clarke *et al*. 2006, Liu *et al*. 2013). These molecular techniques include gel-based identification methods (Pank *et al*. 2001, Farrell *et al*. 2009), DNA barcoding (using the cytochrome oxidase *c* subunit I; Ward *et al* 2008), sequenced-based identification methods (using sequences of the cytochrome *b* (Blanco *et al*. 2008) or NADH dehydrogenase subunit 2 gene regions (Naylor *et al*. 2012)), and high-resolution melting (HRM) analysis (Morgan *et al*. 2011). Furthermore, a few recent studies have demonstrated the suitability of microsatellite markers for species identification based on species-specific allele sizes (Marino *et al.* 2014) and distinctive allele frequencies at multiple loci (Maduna *et al.* 2014; Giresi *et al.* 2015; Marino *et al*. 2017). Although these methods are effective for species identification, most of them involve multiple post-polymerase chain reaction (PCR) manipulations of samples, such as sequencing reactions, restriction digests and gel electrophoresis (Morgan *et al*. 2011). This adds time and cost to the processing of samples, and can be a major disadvantage when processing a large number of samples. Moreover, multiple post-PCR manipulations of samples may increase the chances of human error and contamination (Rugman-Jones and Stouthamer 2016). A more cost-effective and high-throughput approach such as the HRM analysis is more suitable when large numbers of samples are to be processed. Importantly, HMR is a closed-tube assay (meaning less cross-contamination than other methods), and does not require any post-PCR actions (Vossen *et al*. 2009). It involves, first, the incorporation of an intercalating dye that binds to double-stranded DNA (*i.e.*, PCR product) and fluoresces when bound. Second, the double-stranded amplicon is incrementally heated until the DNA duplex dissociates (or melts), while the HRM instrument measures the fluorescence of the reaction. Third, the amplicon begins to denature when heated, resulting in a decrease

in fluorescence (as the intercalating dye is released), and such decreasing fluorescence can be plotted against increasing temperature to produce a melt curve, which is characteristic for that particular amplicon. Last, the first-derivative curves can be converted into melt peak charts through plotting the negative derivative of fluorescence against temperature.

Misidentification of sharks in fisheries operation is a major concern also in South African fisheries (Da Silva *et al*. 2015). Target species include the smoothhounds *Mustelus mustelus*, whitespotted smoothhound *M. palumbes*, tope shark *Galeorhinus galeus*, copper shark *Carcharhinus brachyurus*, dusky shark *Carcharhinus obscurus*, and broadnose sevengill shark *Notorynchus cepedianus* (Da Silva 2007; Da Silva and Bürgener 2007; Da Silva *et al*. 2015). Smoothhounds are commonly misidentified and confused with other triakid species including tope shark and the decommercialised spotted gully shark *Triakis megalopterus*. In an attempt to resolve misidentification issues and enable collection of species-specific data from shark processing plants, Da Silva (2007) developed a dressed demersal identification key for identifying headed and gutted sharks. This identification key includes the number and size of dorsal fins, presence or absence of spine on the first dorsal fine, presence or absence of interdorsal ridge and black/white spots and two morphometric measurements (dorsal origin to caudal tip and dorsal origin to precaudal tip). This approach however requires careful morphological analysis from expert taxonomists before any final decision on the specimen identity can be made. Also, this approach is labour intensive and may present a challenge when screening a large number of specimens. The diagnostic characters used are known to vary with ontogeny (Compagno 1984; Heemstra 1973; Rosa and Gadig 2011; White *et al*. 2013). In light of this, DNA–based specimen identification and morphological analysis should ideally be done in combination to assure best results.

The aim of this chapter is to develop the first molecular toolkit for bridging the gap between fisheries management, law enforcement and conservation genetics in the South African shark demersal fisheries. Here, the goal was to conduct a detailed

comparison of external morphology (headed and gutted) of two commercially important smoothhound species, the common smoothhound *Mustelus mustelus* and whitespotted smoothhound *M. palumbes*, to validate a dressed morphological key proposed by Da Silva (2007). Also, this study aimed to develop molecular identification methods to differentiate between six houndshark species from southern Africa, which include the tope shark *Galeorhinus galeus*, Arabian smoothhound *M. mosis*, common smoothhound, whitespotted smoothhound, flapnose houndshark *Scylliogaleus quecketti* and spotted gully shark *Triakis megalopterus*. These methods include the use of (1) allelic frequency differences between species in a panel of cross-amplified microsatellites, and (2) High Resolution Melt (HRM) analysis of a 16S rRNA gene fragment.

6.2. Materials and methods

6.2.1. Shark tissue and specimen sampling

All reference demersal shark whole-body specimens for morphological analysis of tope shark (N = 12), common smoothhound (N = 32) and whitespotted smoothhound (N = 43) were collected by the Department of Agriculture, Forestry and Fisheries (DAFF), South Africa during demersal longline research surveys off the south–east coast of South Africa in 2016. Test samples including fin and muscle tissues for the tope shark, Arabian smoothhound, common smoothhound, whitespotted smoothhound, flapnose houndshark and spotted gully shark were provided by DAFF or by experienced shark scientists (**Table 6.1**). Attempts at obtaining samples of the Arabian smoothhound within the south-western Indian Ocean (South Africa) were unsuccessful; however, representative samples could be obtained from the north-western Indian Ocean (Oman). Efforts were made to maximise coverage of geographical ranges for each species (**Table 6.1).**

Table 6.1 Summary of the species and the number of individuals (*N*) per species per location included in the present study.

Species	Code	*N*	Locality	Collector
Galeorhinus galeus	Gga	7	Robben Island	DAFF
		7	False Bay	DAFF
		3	Struis Bay	DAFF
		2	Mossel Bay	DAFF
		5	Port Elizabeth	DAFF
Mustelus mosis	Mmo	8	Sultannate of Oman	Mikhail Chesalin
Mustelus mustelus	Mmu	8	Langebaan Lagoon	DAFF
		8	Robben Island	DAFF
		8	False Bay	Michelle Soekoe
		8	Struis Bay	Viking Fisheries
		8	Jeffreys Bay	South African Shark Conservancy
		8	Durban	KZN Sharks Board
Mustelus palumbes	Mpa	11	Yzerfontein	Grant van der Heever
		13	Mossel Bay	Gibbs Kuguru
		16	West Coast	DAFF
Scylliogaleus quecketti	Squ	11	Durban	Bruce Mann
Triakis megalopterus	Tme	8	Cape Point	Michelle Soekoe
		8	Betty's Bay	Michelle Soekoe
		16	Port Elizabeth	Michelle Soekoe

6.2.2. Morphological analysis

For each specimen, the sex was recorded and the flexed total length (L_T, upper caudal fin lobe straightened along the body axis) as well as standard length (L_S) were measured to the nearest 1 millimetre (mm). Additionally, the diagnostic traits proposed for smoothhounds when headed and gutted were recorded, including the dorsal origin to caudal tip length (L_{DOCT}) and dorsal origin to precaudal tip length (L_{DOPCT}), **Figure 6.1**. Statistical analysis was carried out with the statistical software PAST v. 3.16 (PAlaeontological STatistics, Hammer *et al*. 2001). A UPGMA cluster analysis was performed on Euclidean distances of morphometric data (L_{DOCT} and L_{DOPCT}) to show grouping among smoothhound species. A principal component analysis (PCA) was performed on log10-transformed measurements to identify morphological groups based on the combined morphometric data, including the measurements L_{DOCT} and L_{DOPCT}.

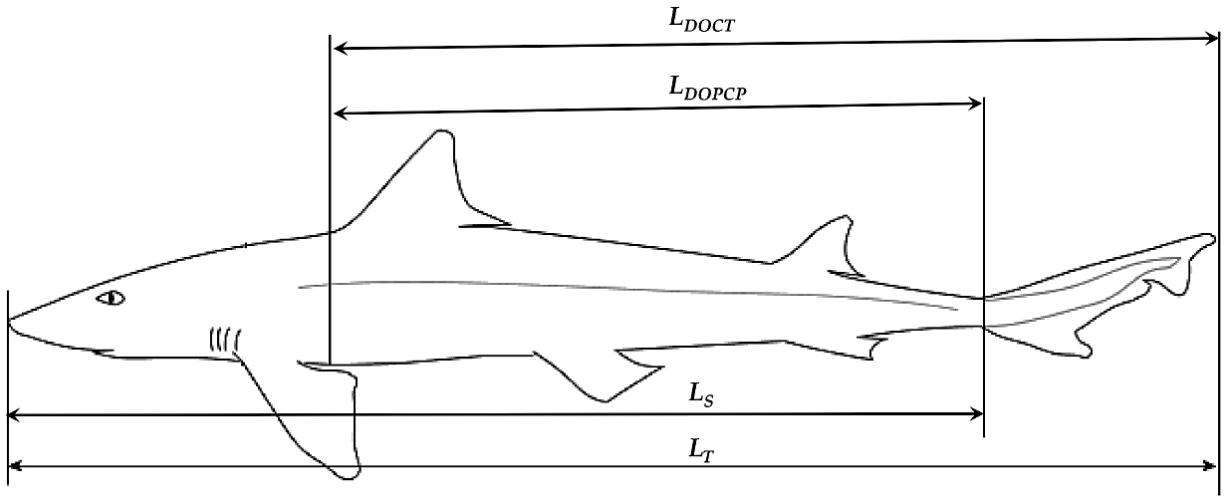

Figure 6.1 Representation of morphometric measurements taken for *Mustelus mustelus* and *M. palumbes*. L_T, total length; L_S, standard length; L_{DOCT}, dorsal origin to caudal tip length; L_{DOPCT}, dorsal origin to precaudal tip length.

Each specimen was dissected with a ventral incision from the cloaca to the pectoral girdle in order to expose the body cavity. For male sharks, maturity was categorised based on the state of the clasper. The classes used were juvenile (*i.e.* claspers undeveloped, not extending beyond posterior tips of pelvic fins), maturing (extending beyond posterior margin of pelvic fin, but flexible and not fully calcified) and mature (much longer than pelvic-fin rear margin, rigid) (Watson and Smale 1998; Stehmann 2002). For female sharks, maturity was assigned following internal examination of the ovary and oocytes, and the development of the nidamental glands and uteri. Females were considered mature when the ovaries were large and well developed (5–34 g). Enlarged yellow oocytes (>3 mm in diameter), that can be easily counted and measured, well developed and vascularized uteri, and large and well developed oviducal glands (width = 16·1–28·8 mm) were also indicators of maturity (Stehmann 2002; Walker 2007). Oocytes and oviducal glands were measured to the nearest mm using a digital calliper.

In addition to the morphometric analyses, general descriptions of dermal denticles were provided for each species. Importantly, these descriptions refer exclusively to the adult stages and thus, given the known ontogenetic variation in these characters, should only be considered for comparisons with other adult specimens. A skin sample of approximately 1 cm^2 was cut from below the dorsal fin and above the lateral line on the left side of the body of each specimen. Residual muscle tissue was removed completely using a scalpel, then the skin sample was cleaned with distilled water and dry stored at room temperature until further analyses. Skin slide preps were examined directly using a light microscope (40–100×) or projected onto a monitor using a digital camera and images captured for further characterisation.

6.2.3. Molecular Analysis

Whenever possible, molecular data was collected from the same individual used to obtain morphological data, especially those used as references specimens for

molecular assay designs. Total genomic DNA was isolated using a modified cetyltrimethylammonium bromide (CTAB) extraction protocol of Sambrook and Russell (2001) as in Chapter 3. The concentration and quality of the DNA were determined by measuring its optical density at 260 nm (A260) and 280 nm (A280) with a NanoDrop ND 2000 spectrophotometer (Thermo Fisher Scientific; wwwthermofisher.com). A subset of samples was subjected to agarose gel electrophoresis for 1 hour at 80 V. The gels were stained with ethidium bromide and photographed under a Gel Documentation system (Gel Doc XR+, Bio-Rad, South Africa).

6.2.3.1. Microsatellite-based species identification

A total of 33 microsatellite loci from previous studies were assessed for their suitability for species identification. Twenty-two microsatellite markers were previously selected from literature, optimised for cross-amplification and characterised in several elasmobranch species by Maduna *et al.* (2014), **Table 6.1**. The remainder of the microsatellites (11) were mined from Next Generation Sequencing (NGS) data and were characterised as described in Chapter 4, **Table 4.2**. As microsatellite data was already available for *Galeorhinus galeus*, *M. mustelus*, *M. palumbes* and *Triakis megalopterus*, the panel was tested for cross-species amplification only in the Arabian houndshark *M. mosis* and flapnose houndshark *Scylliogaleus quecketti* using the previously optimised PCR conditions (Maduna *et al.* 2014). The 33 microsatellites were previously fluorescently labelled with one of the following dyes: FAM, VIC, PET, or NED and optimised into six multiplex assays (5-6 loci per MPS). Multiplex PCR was performed using the Qiagen Multiplex PCR kit according to the conditions specified in Maduna *et al.* (2014). Fragment analysis was performed on an ABI 3730XL DNA Analyzer together with the LIZ600 internal size standard. Individual genotypes were scored based on fragment size in GENEMAPPER v. 4.0 (Life Technologies, South Africa).

Table 6.2 Details of 24 microsatellite markers previously cross-species amplified successfully in southern African houndsharks by Maduna *et al*. (2014), including the primers sequences, microsatellite repeat motif, annealing temperature (T_A) and GenBank accession numbers

Locus	Primer sequence (5′-3′)	Motif	T_A (°C)	Accession number	References
Mh1	F: GGAGGAGGGAAGCCTATGG R: TCTCTGGCTCCATTCAGGG	$(AG)_n$	59	N/A	Chabot (2012)
Mh2	F: ACTACACTGCATATAAACAGGC R: TTTTCAGAGGGCATAACTCAC	$(GA)_n$	56	N/A	Byrne & Avise (2012)
Mh9	F: CAACCATCTTTACTACACTG R: GATGGACCTCACATTTAACAC	$(GA)_n$	56	N/A	Byrne & Avise (2012)
Mh25	F: TGCAATAACCGTTCTGCGTC R: TCACACCCGCAGTTAGATCC	$(CT)_n$	59	N/A	Chabot (2012)
Mca25	F: ACACACTTTCACGCACAAGC R: TCGCTCAAGTGAGACCAGAG	$(CA)_n(CT)_n$	59	JN129145	Giresi *et al*. (2012)
McaB5	F: TAATCGACACGCAGTCATCG R: AAGCTCCAATTCTCACTGTGC	$(GT)_n$	59	JN083996	Giresi *et al*. (2012)
McaB6	F: AGGATAAATACACGCACACAGG R: TTTTTGTTTTGCAATCTCACG	$(CA)_n$	59	JN083997	Giresi *et al*. (2012)
McaB22	F: TCCTCTCCAGGACAAACACAC R: TCCCACCTGCCATAGTAATTG	$(AC)_n$	59	JN083999	Giresi *et al*. (2012)
McaB27	F: ATCCAGTGGTTTTGAAATGC R: CCTCGTAGGTCTCGTC	$(GT)_n$	59	JN129154	Giresi *et al*. (2012)
Mca33	F: CATTTGAACCCCGACAGAAC R: TCCAAGTAAGGATGAGTGACACC	$(ATC)_n$	59	JN083993	Giresi *et al*. (2012)
McaB37	F: TCTGCCTCTGTGTCTCATCC R: TTTCCATTTCCGACATAGGG	$(GT)_n$	59	JN084005	Giresi *et al*. (2012)

Table 6.2 Continued.

Locus	Primer sequence (5′-3′)	Motif	T_A (°C)	Accession number	References
McaB39	F: GGACAGGCAGCATCTGTGTA R: CCCAGGGGGATTAGGATATT	$(CA)_nGAT(AC)_n$	59	JN129156	Giresi *et al*. (2012)
Gg2	F: TGGCTCAGTCCAGAAACCC R: CCCTATTCGAGAGGCCCAG	$(TG)_n$	59	N/A	Chabot & Nigenda (2011)
Gg3	F: CCGTGACTGAAAGCAGCC R: CCCTCAACCATGGCAAGTG	$(GATT)_n$	59	N/A	Chabot & Nigenda (2011)
Gg7	F: CTGTGGAACCAAACTCCAGC R: AGCTGGTCGAGGTGAATGC	$(AG)_n$	59	N/A	Chabot & Nigenda (2011)
Gg11	F: AAGTTGCACGTTTCCCAGC R: TACTGCAGGACCGGTTTCC	$(TCCC)_n$	59	N/A	Chabot & Nigenda (2011)
Gg12	F: TGTCAAACACCATCGCAGG R: TGCTCTGAAGTCTACAAGAATGG	$(TA)_n$	59	N/A	Chabot & Nigenda (2011)
Gg15	F: GGCTGAATGGTTTCCCAGC R: GCCTCCAACTTAGCATAGCC	$(GA)_n$	59	N/A	Chabot & Nigenda (2011)
Gg17	F: CCTGCTTGTGACAGTTACCC R: ACAGGCATCACCTCTGTGC	$(AC)_n$	59	N/A	Chabot & Nigenda (2011)
Gg18	F: TCCACTTCAGGAAGGCCAG R: CAAAGCCAGGTGGTTCTCC	$(GA)_n$	59	N/A	Chabot & Nigenda (2011)
Gg22	F: TCCTGGGATGGCAACTTCG R: AGGCCACCCAACTATCCTG	$(GT)_n$	59	N/A	Chabot & Nigenda (2011)
Gg23	F: ACAGACCACAGGGCATGG R: TGCAGAGCAGGCTAGATGG	$(AC)_n$	59	N/A	Chabot & Nigenda (2011)

To assess whether individuals assigned to distinct groups, ADEGENET was used to perform discriminant analysis of principal components (DAPC) with prior group membership defined by species designation. A Bayesian clustering model-based method implemented in STRUCTURE 2.3 (Pritchard *et al*. 2000) was then used to detect the most probable number of genetic clusters (*K*). A non-admixture model with correlated allele frequencies was applied for 10 replicates across $K = 1$ to $K = 10$ in STRUCTURE v. 2.3 (Pritchard *et al*. 2000) was then used to detect the most probable number of genetic clusters (*K*). A non-admixture model with correlated allele frequencies was applied for 10 replicates across $K = 1$ to $K = 10$ with each run consisting of 1 000 000 Markov Chain Monte Carlo (MCMC) iterations and an initial burn-in phase of 100 000 iterations assuming no prior population information. STRUCTURE HARVESTER v. 0.6.94 (Earl and vonHoldt 2012) was employed to generate averaged likelihood scores for each value of *K* and the *ad hoc* statistic ΔK described in Evanno *et al.* (2005) was used to identify the likely number of genetic clusters. CLUMPAK (Kopelman *et al*. 2015) was used for the graphical representations of the membership assignment plots.

6.2.3.2. High resolution melting analysis-based species identification

To select the best DNA region for high resolution melting analysis, three mitochondrial DNA regions including the 12S-16S ribosomal DNA region (Iglésias *et al*. 2005) and NADH dehydrogenase subunit 2 and 4 (NADH-2 and NADH-4; Boomer *et al*. 2012), were examined for divergent sites at which multiple SNPs or indels exists. These amplicons were also inspected for conserved regions flanking potential SNPs to enable the design of a pair of universal primers. Where necessary each DNA region was amplification using PCR protocols specified in Iglésias *et al*. (2005) and Boomer *et al*. (2012) to generate sequence data. This was followed by sequencing the amplicons using the standard Sanger sequencing chemistry (BigDye® terminator v3.1 cycle sequencing kit). Sequences were edited and aligned using MEGA. Sequences were truncated to shorter fragments (< 190 bp) to include only the region containing

divergent sites and the conserved primer binding sites. Subsequently, *in silico* DNA melting simulations were performed using DNA denaturation models implemented in the program UMELT (Dwight *et al*. 2011). To simulate melting curves, default parameters were used except for the melt temperature range, where the limits were set to 60 and 80 °C, and with increments of 0.25 °C. The UMELT derivative plots were calculated by taking the negative derivative of helicity with respect to temperature. Based on the results obtained, the most suitable gene region for HRM was the 16S rRNA region. Primers were designed for conserved flanking regions of the divergent sites using PRIMER3 v. 0.4.0 (Untergrasser *et al*. 2012).

Initially, for optimisation of the newly designed 16S primer pair, Carch-16S-UniF (5′-AGAAGAGGTACAGCCCTTCTAA-3′) plus Carch-16S-UniR (5′-CCCAATAGGATAAAGGGGTTT-3′), PCR was carried out on a GeneAmp® PCR System 2700 in a 10 µL reaction volume. The latter included 50 ng of template DNA, 1x PCR Buffer, 200 µM of each dNTP, 0.5 µM of each primer, 2.5 mM $MgCl_2$ and 0.5 U of GoTaq® DNA polymerase. The PCR cycling conditions were as follows (i) one cycle of initial denaturation at 95 °C for 2 min (ii) 35 cycles of denaturation at 94 °C for 30 s, 57 °C for 30 s, elongation at 72 °C for 2 min (iii) a final elongation of one cycle at 60 °C for 5 min. Amplification products were then subjected to agarose gel electrophoresis to determine their size.

For HRM assays, PCRs were conducted on a Rotor-Gene Q using the above-mentioned PCR conditions, however adding SYTO® 9 green fluorescent nucleic acid stain at a final concentration of 2.5 µM. This third-generation DNA intercalating dye, SYTO ® 9, can at high concentrations saturate all available sites within double stranded DNA and provides a more accurate assessment of DNA melt status compared to SYBR Green I (Monis *et al*. 2005). It can be used to monitor both the accumulation of the amplified product during PCR and the subsequent product melting on the Rotor Gene Q software v. 2.3.1 (Qiagen). Before HRM, the products were denatured at 95 °C for 5 s, and then annealed at 50 °C for 30 s for DNA duplex formation. HRM was performed as follows: pre-melt at the first appropriate

temperature for 90 s, and melt at a temperature ramping from 65 to 90 °C at 0.1 °C increments every 2 s. The fluorescent data was acquired at the end of each increment step on the FAM channel (excitation at 470 nm, detection at 510 nm) following the 60 °C anneal/extension step. Melting curves were visualised using the Rotor Gene Q software with the digital filter set to zero. The negative derivative of fluorescence (F) over temperature (T) (dF/dt) curve was plotted to display the melting temperature (Tm). Then a normalized raw curve depicting the decreasing fluorescence *vs* increasing temperature was plotted, as well as the difference curves where a specific 'genotype' was used as a baseline. Each species was set as a 'genotype' (reference species) and the average HRM Genotype Confidence Percentages (GCPs) (value attributed to each species being compared to the genotype, with a value of 100 indicating an exact match) for the replicates (disregarding the most outlying replicate) were estimated using the Rotor Gene Q software.

6.3. Results

6.3.1. Morphology

In total, 87 shark specimens/samples from three houndshark species (Arabian smoothhound, $N = 12$; common smoothhound, $N = 32$; whitespotted smoothhound, $N = 43$) were dissected at the Department of Agriculture, Forestry and Fisheries (DAFF) laboratory in Cape Town, South Africa. The overall catch consisted of males and females from juvenile to fully grown individuals, with tope shark comprising seven males and five females (minimum L_T = 936 mm, maximum L_T = 1510 mm), while common smoothhound included 16 males and 16 females (minimum L_T = 1000 mm, maximum L_T = 1590 mm), and *M. palumbes* 28 males and 15 females (minimum L_T = 424 mm, maximum L_T = 830 mm). The dorsal origin to caudal tip length (L_{DOCT}) ranged from 807 mm to 1100 for *M. mustelus* and from 301 to 604 for *M. palumbes*. The dorsal origin to precaudal tip length (L_{DOPCT}) varied from 524 mm to 814 for *M. mustelus* and from 212 to 454 for *M. palumbes*.

The relationship between the L_{DOCT} and L_{DOPCT} were investigated with regression analyses. There was a significant relationship between the dressed morphometric measurements for both species; *M. mustelus* (Pearson correlation: $N = 32$, $R^2 = 0.935$, $P = 0.0001$) and *M. palumbes* (Pearson correlation: $N = 42$, $R^2 = 0.993$, $P = 0.0001$), respectively (**Figure 6.2a**). Separation of the two species was clearly evident from the principal component analysis (PCA), with no overlap observed between species (**Figure 6.2b**). The variation in total length was explained by 99.4 % and 0.6 % for PC1 and PC2, respectively. The UPGMA cluster analysis also revealed the separation between the two smoothhound species (**Figure 6.3**).

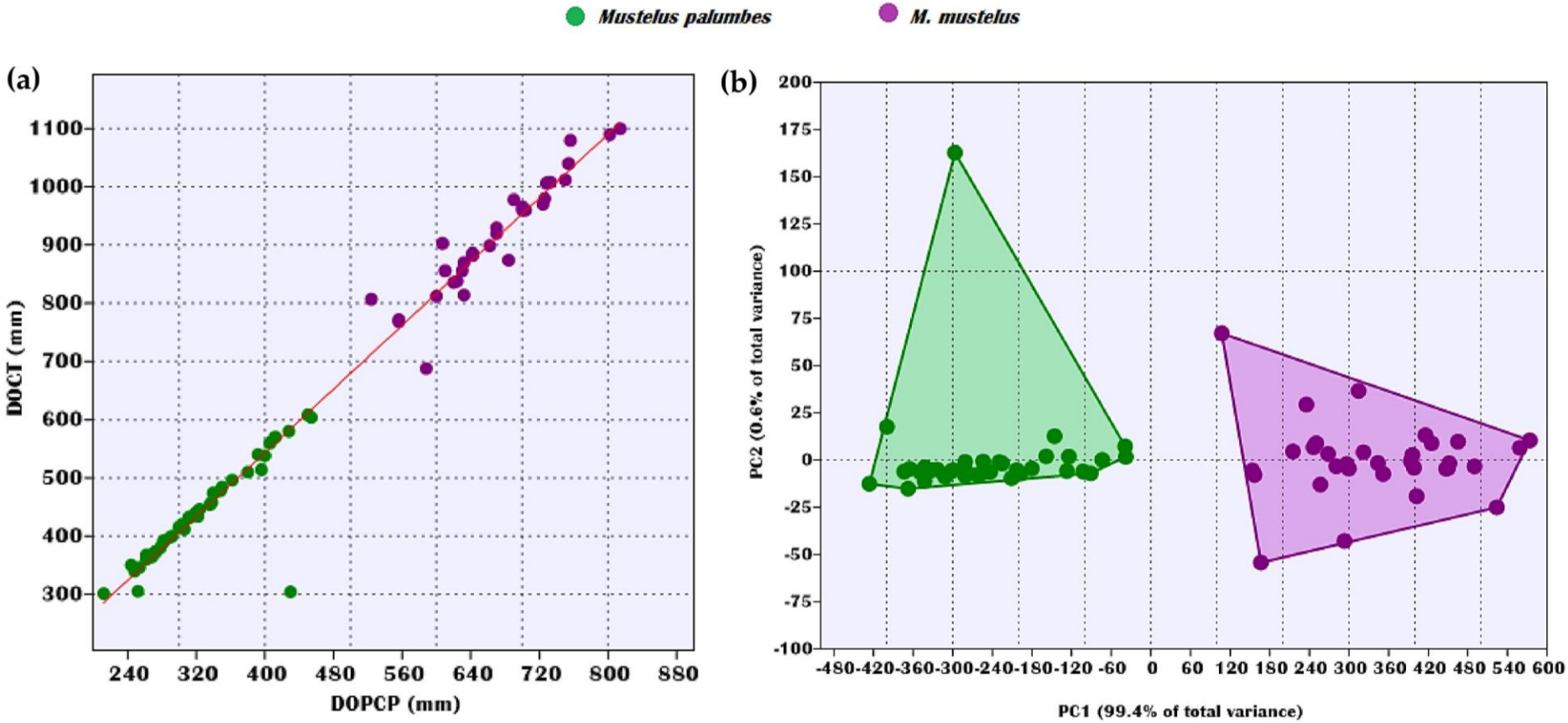

Figure 6.2 Linear regression and principal component analysis of the morphometric measurements used to identify dressed smoothhound sharks, dorsal origin to caudal tip (DOCT) and dorsal origin to precaudal tip (DOPCP).

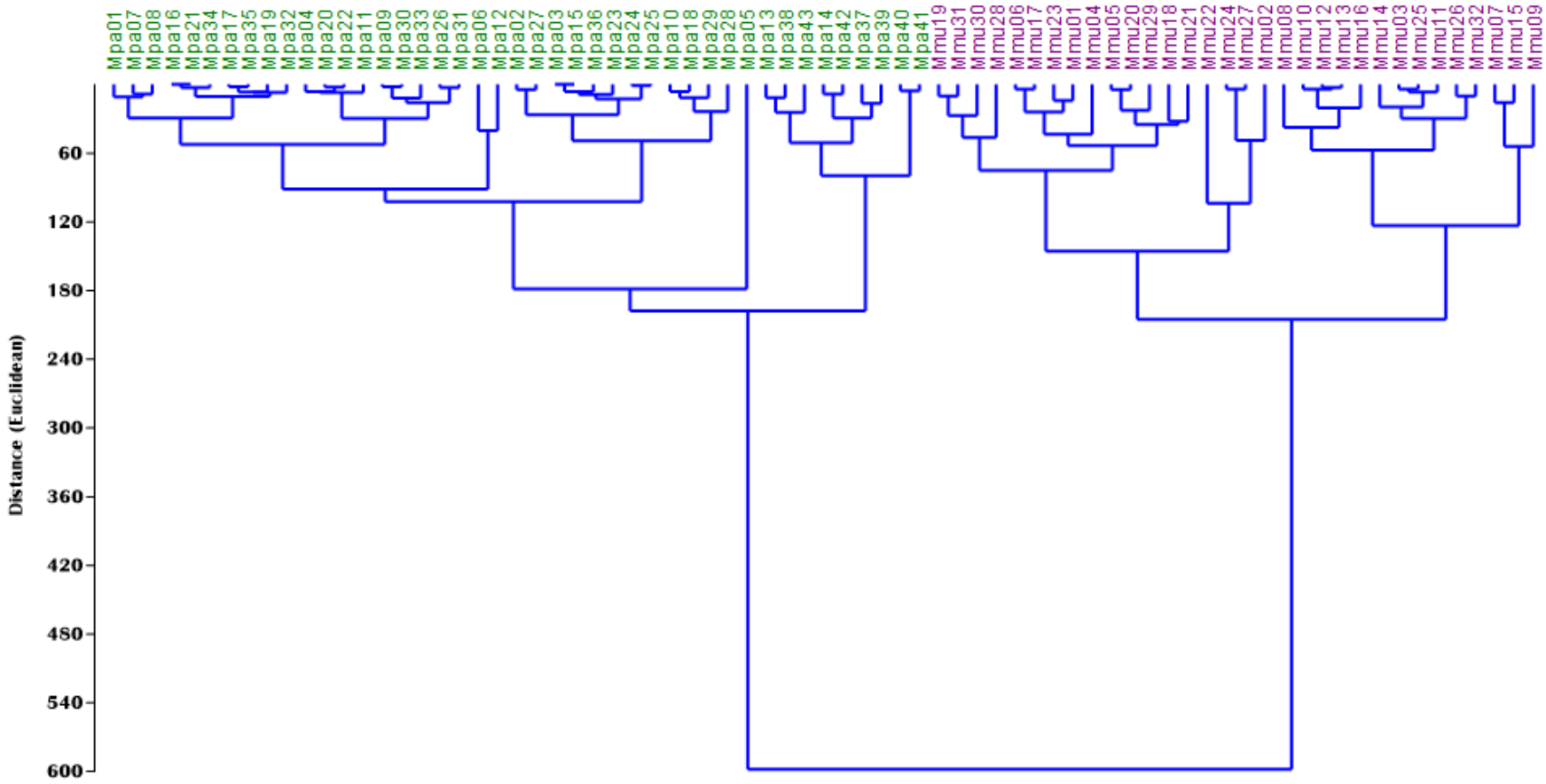

Figure 6.3 UPGMA phenogram of *Mustelus mustelus* (Mmu) and *Mustelus palumbes* (Mpa) based on dressed morphometric measurements.

The examination of the morphology of dermal denticles in the six houndshark species revealed that the denticles contain species-specific characters (**Figure 6.4**).

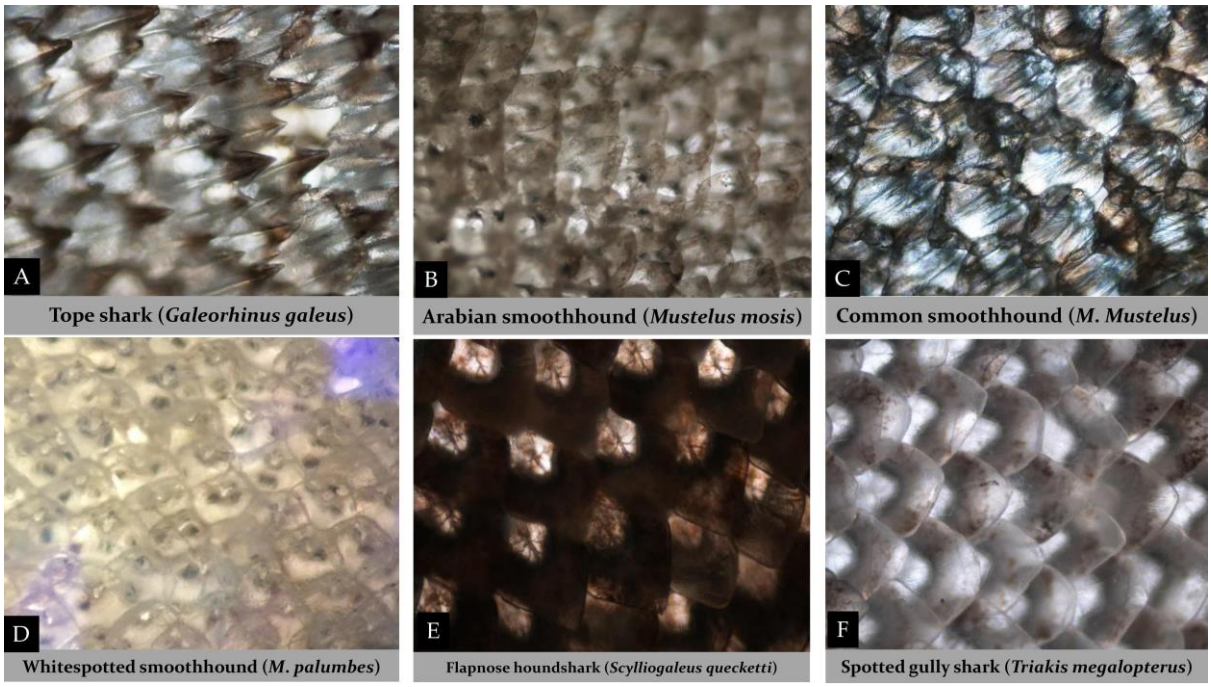

Figure 6.4 Micrographs of dermal denticles from six southern African houndsharks. (A) *Galeorhinus galeus*, (B) *Mustelus mosis*, (C) *M. mustelus*, (D) *M. palumbes*, (D) *Scylliogaleus quecketti* and (E) *Triakis megalopterus*.

For the tope shark, the dermal denticles are densely distributed, and overlap each other along the anterior and lateral margins, posterior margin is more of a "W" shape, and the denticle shape sharp tricuspid (**Figure 6.4A**). For the Arabian smoothhound, the dermal denticles are densely distributed, overlap each other along the anterior and lateral margins, and are a smooth “V” in shape (**Figure 6.4B**). The dermal denticles for the common smoothhound are densely distributed, overlap each other along the anterior and lateral margins, weakly tricuspidate with longitudinal ridges extending along their entire length (**Figure 6.4C**). For the whitespotted smoothhound, the dermal denticles were also densely distributed, overlap each other along the anterior and lateral margins, and are rounded or ellipsoid in shape with no crests (**Figure 6.4D**). For the flapnose houndshark, the dermal denticles are densely distributed, overlap each other along the anterior and lateral margins, and are diamond in shape (**Figure**

6.4E). For spotted gully shark, the dermal denticles are densely distributed, overlap each other along the anterior and lateral margins, and are ellipsoid in shape (**Figure 6.4F**).

What is also of interest is that the smoothhound samples that were identified as the bigeye houndshark (*Iago omanensis*) based on genetic data in Chapter 3 could also be distinguished from the Arabian smoothhound samples based on the dermal denticles (**Figure 6.5**).

Figure 6.5 Micrograph of the bigeye houndshark (*Iago omanensis*) dermal denticles that was misidentified as the Arabian smoothhound (*Mustelus mosis*).

6.3.2. Microsatellite-based species identification

From the 33 microsatellites, only 19 loci were eventually selected for species identification. The microsatellite loci demonstrated potential application in the identification of the species included in this study. The results from the multivariate clustering analysis (DAPC) clearly depict four genetic clusters representative of each species with partial overlap between some species (**Figure 6.6a**). The overlap was mainly as a consequence of the inclusion of highly divergent species, the common smoothhound, flapnose houndshark and spotted gully shark. Upon exclusion of these species, the species (Arabian smoothhound, tope shark and whitespotted smoothhound) that previously overlapped separated into distinct clusters.

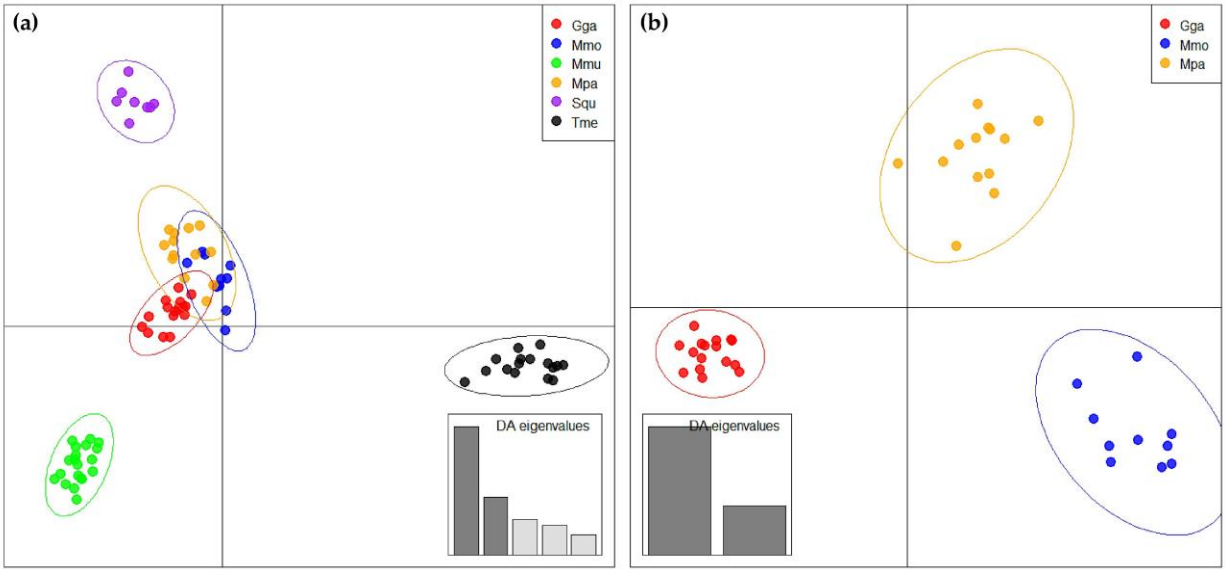

Figure 6.6 a) DAPC analysis based on 19 microsatellite loci including (a) all species and (b) only *G. galeus*, *M. mosis* and *M. palumbes*. See **Table 6.1** for species codes.

The post processing of the STRUCTURE results using the Δ*K* statistic indicated that the most likely value of *K* was 6, corroborating the DAPC results, and that the assignment of individual houndsharks sharks was unambiguous (**Figure 6.7**).

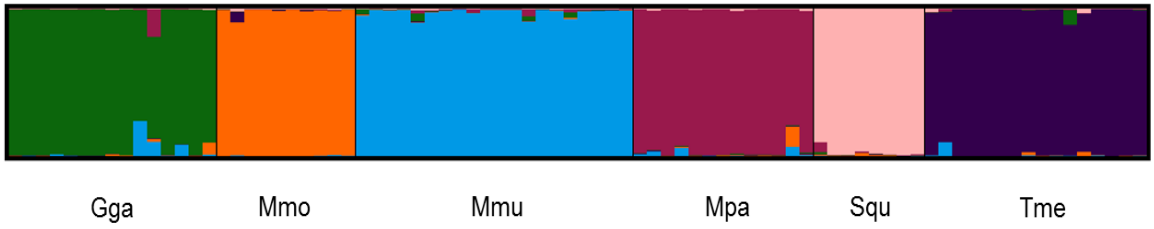

Figure 6.7 STRUCTURE results showing the most likely number of genetic clusters present in each study species. Bar plots showing individual genotype membership to *K* clusters (each cluster is represented by a different colour, and each vertical bar represents an individual). See **Table 6.1** for species codes.

6.3.3. High resolution melting analysis-based species identification

The *in silico* DNA melting simulations using UMELT showed that the 16S rRNA region was the suitable marker for HRM species identification assay. The melting curves predicted in UMELT clearly depicts that individual 16S fragments were characterised by different melting temperatures (**Figure 6.8**).

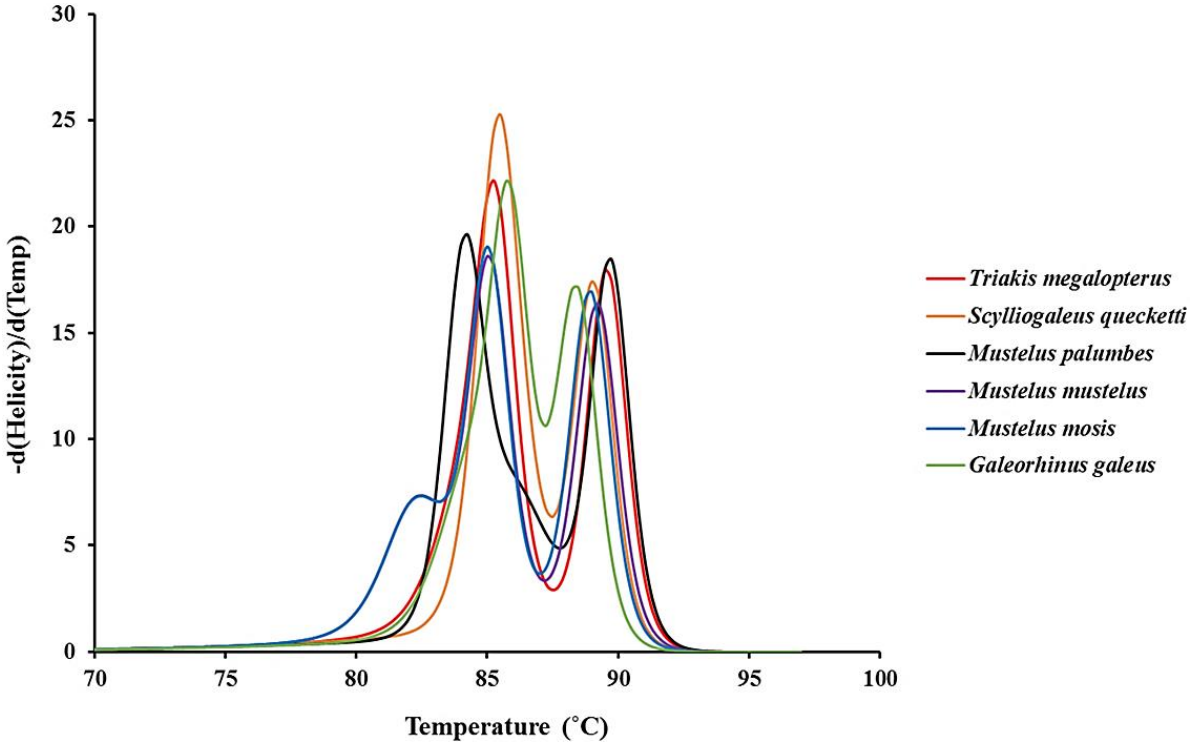

Figure 6.8 Predicted melt peak charts for a fragment of the mitochondrial 16S rRNA gene region for six houndshark species used in the study.

The observed melt profile was also consistent with melting curves predicted in UMELT (**Figures 6.8, 6.9**). The melt profiles generated for different samples within a species were also consistent across replicates (**Figure 6.10**). The average HRM genotype confidence percentages ranged from 91.5% to 100%. The six houndshark species could successfully be distinguished based on the melt profile of the 16S PCR amplicons. The amplicons from all species produced two very distinct melt peaks that were easily scored (**Figure 6.9**).

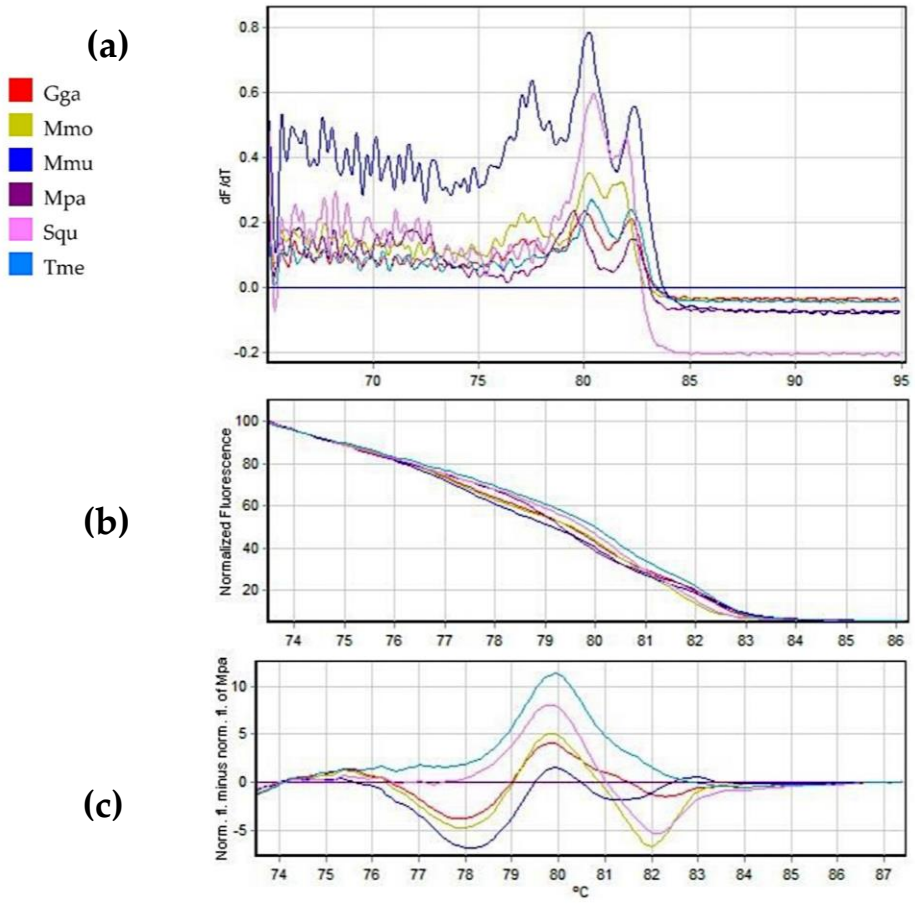

Figure 6.9 High resolution melting (HRM) analysis of the mitochondrial 16S rRNA gene region of houndshark species included in the study. (a) Raw melting profile of *Galeorhinus galeus*, *Mustelus mosis*, *M. mustelus*, *M. palumbes*, *Scylliogaleus quecketti* and *Triakis megalopterus*. (b) Normalization of fluorescence from the raw melting data for further discrimination in a difference plot. (c) Difference plot obtained by using *M. palumbes* as the baseline.

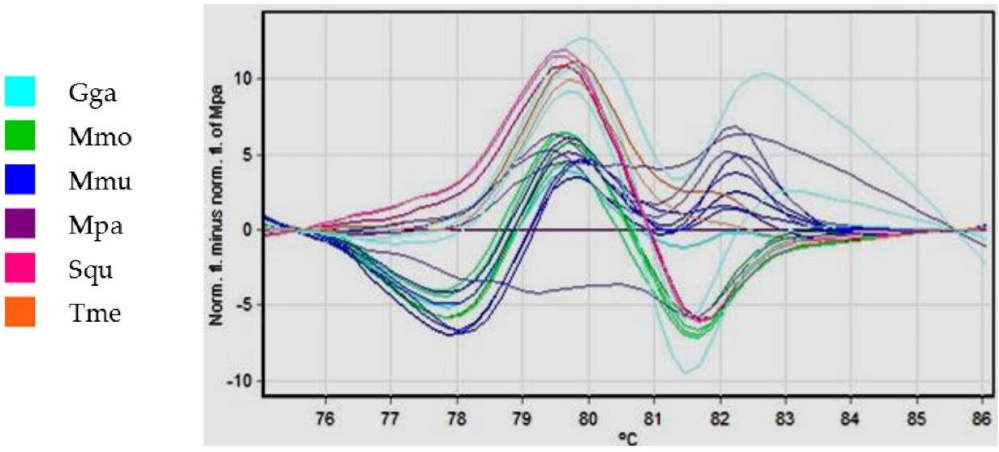

Figure 6.10 The difference melt plot generated in UMELT for replicates using *M. palumbes* as the baseline.

6.4. Discussion

The present study reports the development of molecular assays and morphological features for identifying six houndshark species impacted on by several fishery operations in South Africa (Da Silva *et al*. 2015). A major challenge in the identification of shark species is the overlap of external morphology among species which is exacerbated by the common practice of heading and gutting of sharks at sea by several fisheries (Da Silva 2007; Da Silva and Bürgener 2007). Da Silva (2007) developed a dressed demersal identification key for identifying headed and gutted sharks based on morphological features that are present after processing. This includes two diagnostic morphometric measurements, the dorsal origin to caudal tip length (L_{DOCT}) and dorsal origin to precaudal tip length (L_{DOPCT}). The L_{DOCT} and L_{DOPCT} were applied for the two commercially important species of *Mustelus*, the common smoothhound (*M. mustelus*) and whitespotted smoothhound (*M. palumbes*). The UPGMA cluster analyses and principal component analysis provided clear evidence for the efficacy of the L_{DOCT} and L_{DOPCT} in discriminating between the two South African smoothhounds. These measurements could be used in the field in conjunction with the dermal denticle morphology, an additionally species identification tool identified in the present study. Dermal denticle morphology was diagnostic across the six houndshark species investigated here. Additionally, the Arabian smoothhound (*M. mosis*) could easily be distinguished from the morphologically similar houndshark species, the bigeye houndshark (*Iago omanensis*) solely based on the dermal denticle morphology. Although the dermal denticle morphology may differ between maturity stages (White *et al*. 2013; Veríssimo *et al.* 2014) and between sexes (Crooks *et al*. 2013), the present study confirms the utility of dermal denticle morphology in species identification, as was also evident in previous studies (Gilligan and Otway 2012; Veríssimo *et al.* 2014; Giresi *et al.* 2015; Marino *et al*. 2017). What is promising is that the dermal denticle morphology could possibly also assist in the identification of the study species in cases where only fins are available.

The two molecular approaches (microsatellites-based and high-resolution melting analysis) were found to be suitable for species identification in the current study. The approaches overcame challenges regualary encountered for the routine gel-based idenefication methods such as the lack of specificity of the 'species-specific' primers in spite of *in silico*-based primer specificity testing (Villard and Malausa 2013). For instance, initially, Marino *et al.* (2014) successfully demonstrated the use of the COI and ITS2 to identify the sympatric smoothhounds of the Mediterranean Sea, *M. mustelus* and *M. punctulatus* based on fragment length variance for each species. When the former methods were applied to a larger set of samples in Marino *et al* (2017), the COI and ITS2 assays were not able to assign a fraction of the individuals, 6.6% and 14.4% respectively. The authors attributed the failure of the COI and ITS2 assays in specimen identification to the possible occurrence of heteroplasmy and introgression due to past hybridisation events. Similar to the studies of Marino *et al.* (2017) and Giresi *et al.* (2015), the multiplex assays proved useful in discriminating between the study species, particularly between those that are morphologically very similar. Additionally, the use of standardised panels of microsatellite multiplex PCRs can facilitate the establishment of a genotype database which in future may be useful for conducting comparative population genetics studies as shown in Chapter 4. The high-resolution melting (HRM) analysis has rarely been applied for species identification in sharks (*sensu* Morgan *et al*. 2011). The results of the HRM analysis were consistent with the rest of the methods successfully applied in this study and confirm this tool to be a sensitive technique for distinguishing six houndshark species occurring in southern Africa. This approach could also be applicable to a wide range of houndsharks and other elasmobranch species.

6.5. Conclusion

This chapter reports on the development of morphological and molecular tools for identifying six houndshark species commonly misidentified in the field and

fishery operations in South Africa. The morphometric measurements and dermal denticle morphology could provide fisheries scientists with a relatively simple morphological approach to differentiate between smoothhounds. Dermal denticle morphology can further be used to distinguish all the study species. Given that there is intra-specific variation of denticles, due to growth and different habitats, detailed investigations involving the collection of more specimens across different habitats are warranted. The molecular assay developed in this study for species identification of six houndsharks, which include the use of microsatellites and HRM analysis will complement traditional morphological keys including the dressed morphological key. The implementation of the species identification approaches reported here could form an integral part of collecting species-level data (annual landings, catch rate, and bycatch/discard level) required for basic resource assessments.

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CHAPTER 7

Sperm storage and genetic paternity in the common smoothhound shark: implications for reproductive strategy

Abstract

The reproduction mode of common smoothhound shark *Mustelus mustelus* is placental viviparity and reproduction is seasonal. However, knowledge on the species' reproductive strategy remains poorly understood due to the lack of direct behavioural observations during mating. Several studies in species of *Mustelus* reveal that the frequency of multiple paternity (MP) not only differs between species but also amongst populations and also that a high variability in the relative paternal contribution of males within species exists. These findings hint at a polyandrous mating system, although, it is well known that long-term sperm storage can also result in MP without polyandrous behaviour. This study assessed sperm storage and multiple paternity in common smoothhound shark. Evidence of sperm storage in adult female common smoothhound sharks collected from the south-west coast of South Africa was found and presents the first empirical evidence of sperm storage in *M. mustelus*. In addition, single paternity of a litter of 22 pups is reported based on two microsatellite marker panels (five species-specific- and six cross-amplified microsatellite loci, respectively). Interestingly, no multiple paternity was detected, hinting at the within-species variation in the presence and frequency of MP previously reported for elasmobranchs.

* Please note that this chapter is according to *Journal of Fish Biology* journal requirements

7.1. Introduction

The common smoothhound shark *Mustelus mustelus* (L. 1958) is a medium-sized (total length: <1.7 m) epibenthic member of the houndshark family Triakidae (Compagno 1984; Ebert *et al*. 2013). This species is distributed from the Mediterranean Sea and eastern Atlantic Ocean to the South-West Indian Ocean (Weigmann, 2016). *Mustelus mustelus* is one of the most commercially exploited sharks across its distribution range and is presently classified as 'Vulnerable' in the IUCN Red List of Threatened Species (Serena *et al*. 2009). The reproduction mode of *M. mustelus* is placental viviparity and reproduction is seasonal whereby each cycle may take one year or longer if there is a resting period between pregnancies (Smale and Compagno, 1997; Saïdi *et al*. 2008; Da Silva in prep.). In this species litter size correlates with female size and varies between 2 and 23 pups (Smale and Compagno, 1997; Saïdi *et al*. 2008). Sexual maturity is reached at a total length (L_T) of 70–112 cm for males and 107.5–124 cm L_T for females (Saïdi *et al*. 2008). Overall, sexual maturity, maximum L_T, L_T at birth, litter size and gestation period in *M. mustelus* are known to vary considerably in geographically distant habitats (Smale and Compagno 1997; Capapé *et al*. 2006; Saïdi *et al*. 2008).

Knowledge on the species' mating (copulating) strategy remains poorly understood due to the lack of direct behavioural observations during mating. Marino *et al*. (2015) and Rossouw *et al*. (2016) used bi-parentally inherited microsatellite markers to test the presence or absence of multiple paternity (MP, where a single litter is sired by multiple males) in *M. mustelus* litters and ascribed the presence of the former to a polyandrous mating system (where females mate with multiple males in a single breeding season). Marino *et al*. (2015) reports an overall frequency of 47% (nine out of 19 litters) of MP for *M. mustelus* occurring in the southern Tyrrhenian- and northern Adriatic regions of the Mediterranean Sea, assuming the existence of a single regional stock for the species. In their study, six out of 11 litters (55%) sampled in the

Tyrrhenian region were sired by at least two males, while three out of eight litters (38%) sampled in the northern Adriatic region were sired by multiple males. In Rossouw *et al*. (2016), an even greater percentage of MP is reported for the species in the South-West Indian ocean region (**Figure 7.1**), where MP was determined in four out of six litters (67%). Several studies in species of *Mustelus* reveal that the frequency of MP, measured as the percentage of multiple-sired litters, not only differs between species but also amongst populations of the same species (Byrne and Avise, 2012; Boomer *et al*. 2013; Chabot and Haggin, 2014; Farrell *et al*. 2014; Marino *et al*. 2015; Rossouw *et al*. 2016). Furthermore, a few of these studies also reveal a high variability in the relative paternal contribution of males within species (Boomer *et al*. 2013; Farrell *et al*. 2014; Rossouw *et al*. 2016). These findings hint at a polyandrous mating system in *M. mustelus*, although, it is well known that long-term sperm storage across breeding seasons can also result in MP without polyandrous behaviour within a single breeding season (Pearse *et al*. 2001; Adams *et al*. 2005; Lage *et al*. 2008; Brante *et al*. 2011). In fact, several studies provide evidence for sperm storage in several species of *Mustelus* including *M. antarcticus* (Storrie *et al*., 2008), *M. asterias* (Farrell *et al*., 2014), *M. canis* (Conrath & Musick, 2002; Hamlett *et al*., 2002) and *M. mosis* (Moore *et al*., 2016).

An understanding of a species' mating system is a fundamental requirement for long-term, effective management and conservation strategies, especially when reproductive parameters are geographically variable (Saïdi *et al*. 2008; Park *et al*. 2013; McCully Phillips and Ellis, 2015). To broaden the knowledge base on the reproductive strategy of *M. mustelus*, the aim of this study was firstly to assess sperm storage in the species and then extends on the study by Rossouw *et al*. (2016) to investigate the suggestion of Boomer *et al*. (2013) that sample location should be accounted for during MP assessment to provide information on the frequency of MP between locations.

Figure 7.1 Sampling regions for litters of *Mustelus mustelus* for the present study (1, Mossel Bay) and a previous study (2, coast of KwaZulu Natal) by Rossouw *et al*. (2016).

7.2. Materials and methods

7.3.1. Specimen sampling and DNA Extraction

Two mature female *M. mustelus* specimens were collected under Department of Agriculture, Forestry and Fisheries (DAFF), South Africa research permit during demersal longline research surveys conducted by DAFF off the south–east coast of South Africa in 2016. Sharks were kept in the freezer prior to being dissected at the Department of Agriculture, Forestry and Fisheries (DAFF) laboratory in Cape Town, South Africa. Each specimen was dissected with a ventral incision from the cloaca to the pectoral girdle in order to expose the body cavity. Maturity was assigned following internal examination of the ovary and oocytes, and the development of the nidamental glands and uteri. (Stehmann 2002; Walker 2007). Oocytes and oviducal glands were measured to the nearest mm using a digital calliper. To determine sperm storage, the oviducal glands (OGs) for each specimen were removed and stored in 10% neutral buffered formalin prior to histological examination. For multiple

paternity assessment in *M. mustelus*, fin clip samples were obtained from a single gravid female (1500 mm L_T) with *in utero* litter of 22 pups (100-130 mm L_T, 13 males and 9 females) opportunistically collected by the Oceans Research team in April 2015 at Mossel Bay, an embayment region within the south–west coast of South Africa. The gravid female was found dead at the mouth of the Klein Brak River by the team during their sampling trips.

7.3.2. Oviducal gland histology

The oviducal glands (OGs) were sectioned sagittally, dehydrated through a graded series of alcohol baths (70%-100%,) cleared in Toluene, and embedded in Paraplast Plus paraffin wax (56C). The embedded material was then serially sectioned at 4–7 μm thickness and stained with Harris' hematoxylin and eosin staining. Histological sections were viewed under an Olympus BX60 light microscope (40–100×) and selected sections were projected onto a monitor using an Olympus PM-C35DX digital camera to capture images for further characterisation.

7.3.3. Genetic assessment of multiple paternity

Total genomic deoxyribonucleic acid was isolated using a modified cetyltrimethylammonium bromide extraction protocol of Sambrook and Russell (2001), Chapter 3. The concentration and quality of the DNA were determined by measuring its optical density at 260 nm (A260) and 280 nm (A280) with a NanoDrop ND 2000 spectrophotometer (Thermo Fisher Scientific; wwwthermofisher.com). Individuals were genotyped using two separate microsatellite loci panels; five species-specific loci (*Mmu1*, *Mmu3*, *Mmu7*, *Mmu8* and *Mmu11*) and six cross-amplified microsatellite loci (*McaB6*, *McaB22*, *Gg2*, *Gg22* and *Gg23*) with polymerase chain reaction amplifications performed as outlined in Chapter 4 and Rossouw *et al*. (2016), respectively (**Table 7.1**). Individual genotypes were scored based on fragment size via GENEMAPPER 4.0 (Life Technologies, South Africa).

Table 7.1 Genetic variation descriptors at 11 microsatellite loci grouped into two panels (species-specific and cross-amplified) used for paternity analysis in common smoothhound shark. N_I, Number of individuals scored; N_A, number of alleles; A_E Number of effective alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; *PIC*, polymorphic information content; *PE*, expected exclusion probability when one parent is known with certainty; *PrDM*, probability of detecting multiple paternity given 22 progeny sampled per litter and two fathers contributing 50:50/70:30/ 90:10.

Locus	Reference	N_I	N_A	A_E	H_O	H_E	*PIC*	*PE*	*PrDM*
Species-specific									
Mmu1	Maduna *et al*. 2017	47	7	3.4	0.9574	0.7129	0.6595	0.470	
Mmu3	Maduna *et al*. 2017	46	6	2.6	0.4565	0.6175	0.5449	0.345	
Mmu7	Maduna *et al*. 2017	46	6	1.5	0.3043	0.345	0.3258	0.194	
Mmu8	Maduna *et al*. 2017	45	9	4.8	0.6889	0.802	0.7638	0.599	
Mmu11	Maduna *et al*. 2017	39	4	3.0	0.7179	0.6727	0.5979	0.385	
Overall			**6**	**3.1**	**0.625**	**0.630**	**0.578**	**0.931**	**0.963/0.957/0.796**
Cross-amplified									
McaB5	Giresi *et al*. 2012	48	8	3.6	0.896	0.729	0.681	0.500	
McaB6	Giresi *et al*. 2012	48	6	3.0	0.708	0.669	0.615	0.421	
McaB22	Giresi *et al*. 2012	48	12	8.2	0.896	0.888	0.866	0.755	
Gg2	Chabot and Nigenda, 2011	48	7	2.9	1.000	0.667	0.604	0.407	
Gg22	Chabot and Nigenda, 2011	47	3	2.3	0.957	0.564	0.458	0.259	
Gg23	Chabot and Nigenda, 2011	48	6	2.8	1.000	0.645	0.571	0.365	
Overall			**7**	**3.8**	**0.910**	**0.694**	**0.632**	**0.980**	**0.994/0.992/0.864**

Baseline allele frequencies were obtained for each microsatellite multiplex panel from a population data set consisting of 48 adult sharks collected from the South-West Indian Ocean given the interoceanic population genetic structure determined for common smoothhound (Maduna *et al.*, 2014; 2017). All loci were evaluated for scoring errors and allelic dropout using MICROCHECKER 2.2.3 (van Oosterhout *et al*. 2004). CERVUS 3.0.7 (Kalinowski *et al*. 2007) was used to assess genetic variation descriptors at single loci and across all loci by calculating the number of alleles (N_A), estimating observed (H_O) and expected (H_E) heterozygosities and polymorphic information content (*PIC*). Additionally, the effective number of alleles (A_E) was calculated using GENALEX 6.5 (Peakall and Smouse 2012) and the expected exclusion probabilities with GERUD 2.0 (Jones, 2005). Since the power to detect multiple paternity depends on the level of marker polymorphism, the statistical power of genetic analysis to detect mixed paternity litters was assessed using PrDM (Neff and Pitcher, 2002). Four different scenarios were run for the PrDM software: (i) two sires with equal contributions (0.5:0.5), (ii) two sires with moderately skewed contributions (0.7:0.3) and (iii) two sires with highly skewed contributions (0.9:0.1). Finally, GERUD was employed to estimate the minimum number of fathers in the single litter *i.e*. presence or absence of multiple paternity.

7.3. Results and Discussion

Within the sectioned oviducal glands, sperm was found in the lumen of the terminal zone as well as in sperm storage tubules (SSTs) as a mass of densely packed and non-aligned spermatozoa (**Figure 7.2**). Histological observations also revealed the presence of spermatozoa between the sloughed cells in the lumen of the OG suggesting either a recent mating or the release of stored sperms. The study specimens were collected prior to the austral spring and summer breeding seasons of South African *M. mustelus* (Smale and Compagno, 1997). All things considered, the sampling strategy and the results give substantial confidence that *M. mustelus* has the capability

to store sperm, although it remains unclear whether spermatozoa are stored short-term (weeks to months) or long-term (across multiple breeding seasons). Nevertheless, the considerable number of SSTs observed and their deep localization suggest possible long-term sperm storage ability in *M. mustelus*, which has been observed in other shark species (Pratt, 1993; Storrie *et al*. 2008; Farrell *et al*. 2010), Fig. 2. Although long-term sperm storage could result in multiple paternity without polyandrous behaviour within a single breeding season, it remains speculative for species of *Mustelus* and other elasmobranch taxa (Farrell *et al*. 2014; Marino *et al*. 2015).

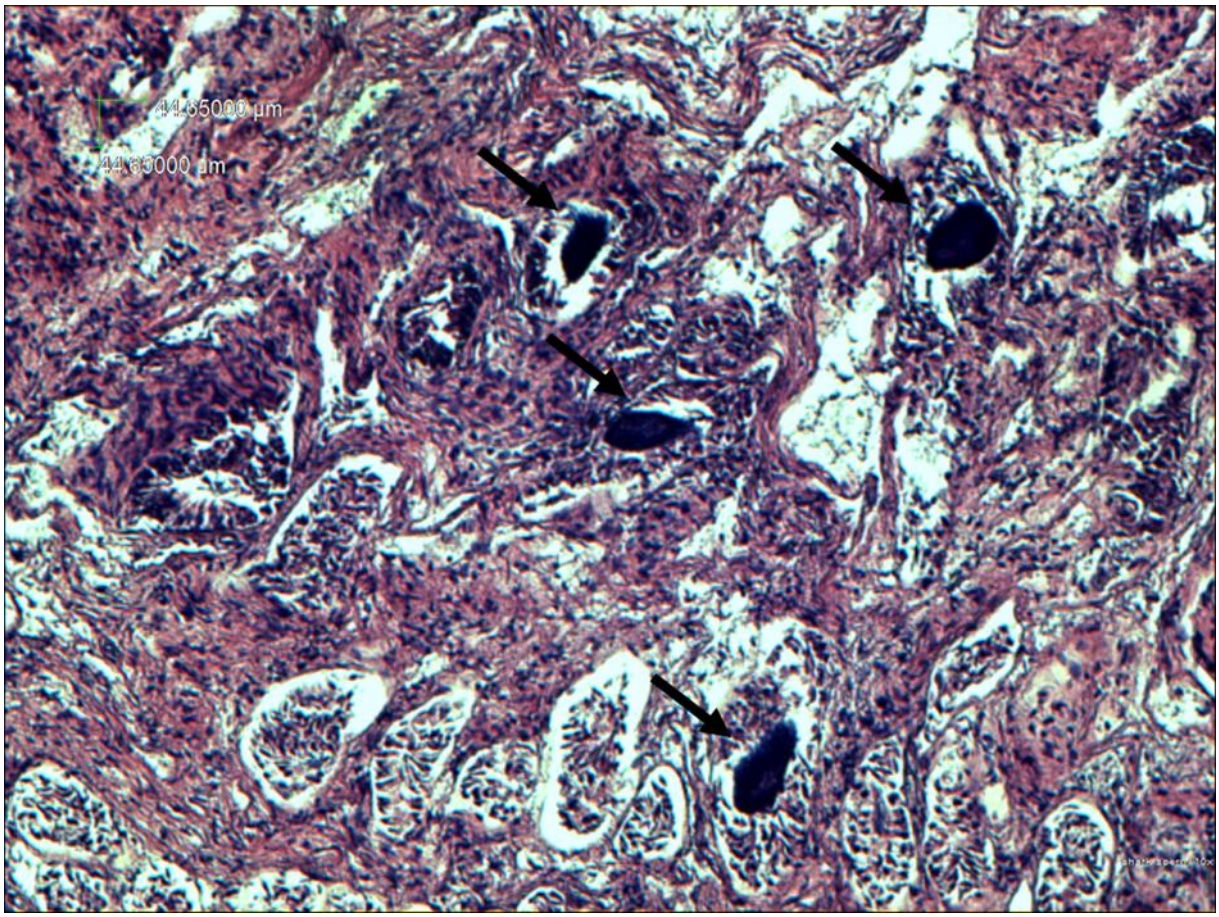

Figure 7.2 Sperm storage tubules (designated by arrows) containing sperm bundles, as observed in the terminal zone of the oviducal gland of adult female common smoothhound shark.

All the microsatellites used in this study were polymorphic with N_A ranging from of 2 to 4, A_E ranging from 1.4 to 4, H_E and *PIC* as high as 0.764, as well as provided

considerable power for detecting multiple paternity with no difficulties in scoring (**Table 7.1**). No multiple paternity was detected in the litter from Mossel Bay with either of the cross-species amplified or the species-species microsatellite panels used. Here it is argued that the single paternity (*i.e.* genetic monogamy) detected in the current study is neither an artefact of marker properties nor litter size since this is the largest litter analysed for paternity to date for the species. In general, sharks exhibit complex genetic mating systems with considerable interspecific variability (Chapman *et al*. 2004, 2013), however, the genetic monogamy detected in the current study is interpreted as an indication of possible geographic variation in the frequency of MP in South African *M. mustelus*, as was evident in the Mediterranean *M. mustelus* (Marino *et al*. 2015). This trend has previously been documented in the brown smoothhound shark *Mustelus henlei* by Chabot and Haggin (2014). Further investigations of MP in a larger sample size (in terms of the number of litters) will enable a more accurate estimate of the frequency of MP in the Mossel Bay region. Furthermore, given that *M. mustelus* aggregates in social groups to some extent (Compagno, 1984; Smale and Compagno, 1997), it is proposed that the data will provide a baseline for detecting shifts in operational sex ratios *i.e*., the ratio of breeding males to females (Emlen and Oring 1977). Shifts in operational sex ratio have previously been shown to affect mating behaviours and mate choice, depending on the more abundant sex (Jouventin *et al*. 2007; Stewart and Dutton, 2014).

The current study represents the first empirical evidence of sperm storage in *M. mustelus*. Future work will be focusing on assessing sperm storage in the entire reproductive tract throughout the reproductive cycle of the species and estimating the frequency of MP in embayment regions of South Africa. The aspects of *M. mustelus* reproductive strategy reported here are all important for a more holistic and biologically meaningful approach to the management and conservation of this globally exploited shark species.

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CHAPTER 8

General discussion: main findings and future directions

8.1. What have we learnt and what could we learn in the future?

The research presented in this dissertation aimed to address crucial issues vital to the management and conservation of commercially important smoothhound shark species using a multidisciplinary approach. Importantly, the results presented in this dissertation advanced the knowledge base on the shark genus *Mustelus*, particularly for species distributed in the southern African region. In Chapter 2, a critical and complete synthesis of the literature spanning three decades of molecular research on the shark genus *Mustelus* revealed new paradigms and the existence of knowledge gaps. The main areas identified included the phylogenetic biogeography of the genus *Mustelus*, population genetics parameters (*e.g.*, gene flow and effective population size), molecular species identification and, reproductive behaviour and strategy. As such, to address these gaps in knowledge a multidisciplinary approach was recommended, that included the use of molecular-, next-generation sequencing-, morphometric- and histologic approaches.

Members of the *Mustelus* genus are uniquely distributed across ocean basins with a high degree of sub-regional endemism. A regional species-level molecular phylogeny of *Mustelus* was constructed using one nuclear gene (KBTBD2) and three mitochondrial DNA genes (CR, 12S-16SrRNA and NADH-2) using a statistical historical biogeographic approach (Chapter 3). First, the inferred phylogenetic revealed the phylogenetic position of the north-eastern Atlantic endemic, the blackspotted smoothhound *M. punctulatus* and, the placement of the spotted gully

shark *Triakis megalopterus* as a highly divergent ancestral species of *Mustelus*. Second, based on the statistical historical biogeographic results, there is conclusive evidence for the radiation of *Mustelus* in the southern African region primarily driven by long-distance dispersal. The observed paraphyly of southern African *Mustelus* species also suggested that there are possibly two separate colonisation events from the Northern Hemisphere. A third and separate colonisation event for the isolated population of the Arabian smoothhound *M. mosis* is highly likely, and this could in future be verified if samples from the east coast of South Africa are included.

In general, even in the genomics revolution, many shark species remain devoid of molecular genetic resources necessary for basic population genetic and demographic assessment. In chapter 4, a set of *in silico* NGS mined microsatellites were characterised in the common smoothhound and tested for cross-species amplification in confamilial triakid species. Likewise, using a customised bioinformatics pipeline, single-nucleotide-polymorphism (SNP) were developed for the common smoothhound and tested for cross-species amplification in the congeneric whitespotted smoothhound *M. palumbes* (Chapter 5). Chapter 5 provided a framework for a SNP discovery approach, *de novo* SNP identification and KASP SNP genotyping technology, which should be transferable and useful for other non-model shark species in future. In chapter 4, the transferability of microsatellite loci enabled the development of standardised panels of microsatellite multiplex PCRs for comparative population genetics studies and molecular identification of species. The comparative population genetics of South African species of *Mustelus*, and two coastal houndsharks, the tope shark *Galeorhinus galeus* and spotted gully shark *Triakis megalopterus* implied that combined effects of oceanographical barriers and life-history differences (*e.g.* mobility and sex-specific dispersal strategies) are responsible for shaping regional population structure in these sharks. Even though the sampling populations were of limited size, the similar pattern of genetic structuring observed across these species is a step towards a pragmatic multispecies management approach. However, it is recommended that it is used to complement rather than replace stock assessment data (in the classical fisheries sense).

Multispecies management of commercially important shark species is largely attributed to the prominence of misidentification issues of sharks owing to morphological conservatism. In order to assist identification of sharks in fishery operations in South Africa, morphological and molecular tools were optimised for six houndshark species (*Galeorhinus galeus*, *Mustelus mosis*, *M. mustelus*, *M. palumbes*, *Scylliogaleus quecketti* and *Triakis megalopterus*) in Chapter 6. It is expected that the species identification methods reported here will be useful for collecting species-level annual landings, catch rate, and bycatch/discard level data required for stock assessments. This in turn can provide useful information to better guide management and conservation of these species across the southern African coast.

Although the reproductive biology of commercially important smoothhounds in South Africa is known (Smale and Compagno 1997; Da Silva in prep), and multiple paternity has been confirmed in common smoothhound (*sensu* Rossouw *et al*. 2016), it is known that populations of the same species may be subjected to different regional selection pressures which may alter their life histories. Also, a number of studies show that the reproductive parameters (maximum total length, total length at birth and maturity, litter size and gestation period) and the frequency of multiple paternity in common smoothhound varies geographically (Smale and Compagno 1997; Capapé *et al*. 2006; Saïdi *et al*. 2008; Marino *et al*. 2015; Rossouw *et al*. 2016; Da Silva in prep). Consequently, the life-history information of a species in one location cannot be assumed to be representative of other populations from different regions (White and Sommerville 2010). In Chapter 7 an attempt was therefore made to investigate a single litter obtained from a different location than that of Rossouw *et al*. (2016) and interestingly, multiple paternity could not be confirmed in this litter. Additionally, Chapter 7 provides empirical evidence for sperm storage in common smoothhound and raises further questions on the polyandrous behaviour of female smoothhounds and the impact of such behaviour on the frequency of multiple paternity. Importantly, sperm storage can be among the factors involved in post-copulatory selection, in this case, through cryptic female choice or by facilitating sperm competition (Fitzpatrick

et al. 2012). The observed sperm storage in the common smoothhound in this study once again highlights the importance of taking biological traits into consideration when interpreting genetic data.

In summary, the research presented in this dissertation have broadened the understanding of smoothhound molecular phylogenetics, biogeography, the distribution of intra-specific genetic diversity, molecular taxonomy and, reproductive biology and genetics on a regional level. This could hopefully provide guidance for further studies using for example genome-wide SNPs permitting accurate stock structure assessment or identification of the most vulnerable stocks. In addition, a broader assessment of population genetic structure is suggested for the wide-spread common smoothhound to ultimately inform management and conservation on a more global scale. Nonetheless, there is a crucial lesson in this throughout, when doing assessments on a molecular level, it is important to place genetic results in a broader context by assimilating biological and ecological data if definitive conclusions are to be drawn.

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Appendix

Published Papers

Maduna SN, Rossouw C, Da Silva C, Soekoe M, Bester-van der Merwe AE. 2017. Species identification and comparative population genetics of four coastal houndsharks based on novel NGS mined microsatellites. 2017. *Ecology and Evolution* 7: 1462–1486.

Received: 16 May 2016 | Revised: 14 November 2016 | Accepted: 21 December 2016

DOI: 10.1002/ece3.2770

ORIGINAL RESEARCH

WILEY Ecology and Evolution Open Access

Species identification and comparative population genetics of four coastal houndsharks based on novel NGS-mined microsatellites

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National Research Foundation of South Africa.

Abstract

The common smooth-hound (*Mustelus mustelus*) is the topmost bio-economically and recreationally important shark species in southern Africa, western Africa, and Mediterranean Sea. Here, we used the Illumina HiSeq™ 2000 next-generation sequencing (NGS) technology to develop novel microsatellite markers for *Mustelus mustelus*. Two microsatellite multiplex panels were constructed from 11 polymorphic loci and characterized in two populations of *Mustelus mustelus* representative of its South African distribution. The markers were then tested for cross-species utility in *Galeorhinus galeus*, *Mustelus palumbes*, and *Triakis megalopterus*, three other demersal coastal sharks also subjected to recreational and/or commercial fishery pressures in South Africa. We assessed genetic diversity (N_A, A_R, H_O, H_E, and PIC) and differentiation (F_{ST} and D_{est}) for each species and also examined the potential use of these markers in species assignment. In each of the four species, all 11 microsatellites were variable with up to a mean N_A of 8, A_R up to 7.5, H_E and PIC as high as 0.842. We were able to reject genetic homogeneity for all species investigated here except for *T. megalopterus*. We found that the panel of the microsatellite markers developed in this study could discriminate between the study species, particularly for those that are morphologically very similar. Our study provides molecular tools to address ecological and evolutionary questions vital to the conservation and management of these locally and globally exploited shark species.

KEYWORDS

cross-amplification, *Galeorhinus galeus*, Illumina sequencing, microsatellites, *Mustelus mustelus*, *Mustelus palumbes*, *Triakis megalopterus*

1 | INTRODUCTION

Sharks play a crucial role in maintaining the ecological balance in marine ecosystems as keystone species, yet these animals are gradually declining worldwide in seascapes heavily impacted by humans (Dulvy et al., 2014). Such declines in wild populations not only will have negative ecological impacts on lower trophic species (Price, O'Bryhim, Jones, & Lance, 2015) but can also alter the levels and distribution of

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genetic diversity among populations (Dudgeon et al., 2012). It is likely that sharks may not respond well to population declines compared to other marine fishes owing to their *K*-selected life-history traits, *i.e.*, slow growth, late maturity, and low reproductive outputs (Compagno, 1984; Ebert, Fowler, Compagno, & Dando, 2013). This highlights the need for conservation and management measures to ensure the sustainable utilization of these fishery resources. Implementing such measures often requires information on fishery dynamics, biological and baseline ecological data which in most cases are not yet available (Velez-Zuazo, Alfaro-Shigueto, Mangel, Papa, & Agnarsson, 2015). Molecular approaches have been very useful in providing insight into historical and contemporary demography of various commercially important shark species, especially with respect to population connectivity, stock structure, and metapopulation dynamics (Boomer, 2013; Chabot, Espinoza, Mascareñas-Osorio, & Rocha-Olivares, 2015; Pereyra, García, Miller, Oviedo, & Domingo, 2010; Sandoval-Castillo & Beheregaray, 2015).

Despite ongoing sampling difficulties, population genetics studies of bio-economically important sharks are now fast increasing due to molecular genetic markers becoming more readily available. For example, next-generation sequencing (NGS) has become a common approach to developing microsatellites in nonmodel organisms as it enables the recovery of thousands of repeat-containing sequences at a reduced time and cost (Blower, Corley, Hereward, Riginos, & Ovenden, 2015; Chabot & Nigenda, 2011; Pirog, Blaison, Jaquemet, Soria, & Magalon, 2015). Also, newly developed microsatellites for source species can be assessed for cross-species transferability in congeneric and confamilial (target) species and have shown to have a high success rate in elasmobranchs (Blower et al., 2015; Boomer & Stow, 2010; Chabot, 2012; Maduna, Rossouw, Roodt-wilding, & Bester-van der Merwe, 2014; Pirog et al., 2015). This allows for the development of a standardized panel of microsatellite multiplex PCRs for comparative population genetics studies and identification of species.

Identification of bio-economically important sharks during port inspections is very difficult (or even impossible) when using traditional taxonomic tools because of carcass processing at sea, where the head and fins are removed (Abercrombie, Clarke, & Shivji, 2005; Akhilesh et al., 2014; Stevens, 2004). During processing morphological and meristic criteria which are pivotal to the accurate identification of specimens are lost (Mendonça et al., 2010; da Silva & Bürgener, 2007). Several different genetic identification methods have previously been developed to resolve misidentification issues (Blanco, Pérez-Martín, & Sotelo, 2008; Naylor et al., 2012; Ward, Holmes, White, & Last, 2008) or to reveal captures of threatened shark species (Clarke et al., 2006; Liu, Chan, Lin, Hu, & Chen, 2013; Shivji, Chapman, Pikitch, & Raymond, 2005). These include gel-based identification methods (Farrell, Clarke, & Mariani, 2009; Pank, Stanhope, Natanson, Kohler, & Shivji, 2001), DNA barcoding (using the cytochrome oxidase *c* subunit I; Ward et al., 2008), sequenced-based identification method (using sequences of the cytochrome *b*; (Blanco et al., 2008) or NADH dehydrogenase subunit 2 gene regions (Naylor et al., 2012)), and high-resolution melting analysis (Morgan et al., 2011). Furthermore, a few studies have recently demonstrated the applicability of cross-species microsatellites for species identification based on species-specific allele sizes (Marino et al., 2014) and distinctive allele frequencies at multiple loci (Giresi et al., 2015; Maduna et al., 2014).

South Africa is an ecologically and evolutionarily dynamic region with a diverse elasmobranch fauna (Bester-van der Merwe & Gledhill, 2015; Compagno, 1984; Ebert et al., 2013) and is located in the transition zone between the Atlantic and Indo-Pacific biomes (Briggs & Bowen, 2012). The Atlantic/Indian Ocean boundary in this region is characterized by two ocean basins, the Southeast Atlantic Ocean (SEAO) and Southwest Indian Ocean (SWIO) with two major currents, the cold Benguela Current and the warm Agulhas Current (Briggs & Bowen, 2012; Hutchings et al., 2009). Thus far, only a few regional population genetics studies related to sharks have been conducted in southern Africa but have shed some light on the possible impact of oceanographic features on gene flow patterns of species affected by fisheries, including the tope shark (*Galeorhinus galeus*), common smooth-hound (*Mustelus mustelus*), and spotted gully shark (*Triakis megalopterus*) (Bitalo, Maduna, da Silva, Roodt-Wilding, & Bester-van der Merwe, 2015; Maduna, da Silva, Wintner, Roodt-Wilding, & Bester-van der Merwe, 2016; Soekoe, 2016). These studies showed that the interaction between the two ocean currents plays a prominent role in limiting dispersal around the southern tip of Africa, particularly in an eastward direction for the common smooth-hound shark for example. Given that single-species conservation strategies do not adequately protect the biological and ecological needs of multiple species within threatened ecosystems, the focus has shifted toward multispecies approaches.

The local distribution ranges of all the triakid species (family Triakidae) investigated here, the tope shark, common smooth-hound, whitespotted smooth-hound (*M. palumbes*), and the spotted gully shark, extend across the Atlantic/Indian Ocean boundary. This presents an ideal opportunity to test whether the interplay of oceanographic features and life-history traits are the drivers of population subdivision in these sharks. The tope shark is a highly mobile semipelagic demersal species that is widely distributed in temperate waters (Ebert et al., 2013). Although sexual maturity depends on the ocean basin of origins, females reach sexual maturity at a total length (L_T) of 118–150 cm and males at 107–135 cm L_T. Reproduction is viviparous (no yolk-sac placenta) with a triennial reproductive cycle (Ebert et al., 2013; Lucifora, Menni, & Escalante, 2004; McCord, 2005). Conversely, smooth-hounds are relatively small and less mobile epibenthic sharks (<170 cm L_T) (da Silva et al., 2013; Smale & Compagno, 1997). The common smooth-hound (Figure 1) is a cosmopolitan species distributed across the Mediterranean Sea, the eastern Atlantic Ocean, and the Southwest Indian Ocean, whereas the whitespotted smooth-hound is endemic to southern Africa and is found from Namibia to northern KwaZulu-Natal (Ebert et al., 2013; Smale & Compagno, 1997). Reproduction in the common smooth-hound is characterized by placental viviparity and a seasonal reproductive cycle whereby each cycle may take 1 year or longer. Sexual maturity is reached at 70–112 cm L_T for males and 107.5–124 cm L_T for females (Saïdi, Bradaï, & Bouaïn, 2008; Smale & Compagno, 1997). For the whitespotted smooth-hound, reproduction is characterized by aplacental viviparity and an aseasonal reproductive cycle although the timing of

FIGURE 1 *Mustelus mustelus*. An individual of *M. msutelus* with evident black spots on the dorsal surface. Picture by Rob Tarr

reproductive cycles is presently unclear. Sexual maturity is reached at 75–85 cm L_T for males and 80–100 cm L_T for females (Ebert et al., 2013; Smale & Compagno, 1997). Similar to smooth-hounds morphologically but with a larger body size, the spotted gully shark is endemic to southern Africa and is found from southern Angola to Coffee Bay, South Africa. Reproduction is ovoviviparous with a biennial to triennial reproductive cycle (Smale & Goosen, 1999; Soekoe, 2016). Sexual maturity is reached at 94–130 cm L_T for males and 140–150 cm L_T for females. Anecdotal evidence based on tagging data suggests that the spotted gully sharks exhibit a high degree of site fidelity or residency because *ca.* 80% of these animals were recaptured close to their release site (within a 20-km radius), regardless of the time at liberty (Dunlop & Mann, 2014; Soekoe, 2016).

Here we characterize a set of NGS-mined microsatellites in common smooth-hound and evaluate the potential of cross-species utility of these markers in species identification and assessing the distribution of genetic variation across populations sampled along the South African coast.

2 | MATERIALS AND METHODS

2.1 | Sample collection and genomic DNA extraction

A total of 144 finclip samples from four coastal shark species (the tope shark, common smooth-hound, whitespotted smooth-hound, and the spotted gully shark) were examined (Table 1). We included samples from the west and east coasts, representing the two main ocean basins (SEAO and SWIO) spanning the South African coastline (Figure 2). The west coast samples represent SEAO individuals collected west of the Atlantic/Indian Ocean boundary, while the east coast samples represent SWIO individuals collected east of the Atlantic/Indian boundary. In addition, we obtained tissues samples from three individuals each of the starry smooth-hound (*Mustelus asterias*) and the blackspotted smooth-hound (*M. punctulatus*) from the Mediterranean Sea, and two individuals of the hardnose smooth-hound (*M. mosis*) from Oman in the northwestern Indian Ocean. Total genomic DNA was isolated using a standard cetyltrimethylammonium bromide (CTAB) extraction protocol of Sambrook and Russell (2001). The concentration and the quality of the extracted DNA were determined by measuring its optical density at 260 nm (A_{260}) and 280 nm (A_{280}) with a NanoDrop ND 2000 spectrophotometer (Thermo Fisher Scientific; wwwthermofisher.com). A small subset of samples was subjected to electrophoresis in 1× TAE buffer for 1 hr at 80 V. Five microliters of the isolated genomic DNA was loaded on 0.8% agarose gel stained with ethidium bromide to check DNA quality. The gels were photographed under a Gel Documentation system (Gel Doc XR+, Bio-Rad, South Africa).

TABLE 1 Details of the sampling locations and sample sizes (*N*) of four coastal shark species

Species	Ocean basin	Collection site	Geographic coordinates	*N*
Mustelus mustelus (*N* = 48)	SEAO	Langebaan Lagoon	33°09′S, 18°04′E	8
		Robben Island	33°48′S, 18°24′E	8
		False Bay	34°10′S, 18°36′E	8
	SWIO	Struis Bay	34°47′S, 20°03′E	8
		Jeffreys Bay	34°35′S, 24°56′E	8
		Durban	29°44′S, 31°07′E	8
Mustelus palumbes (*N* = 40)	SEAO	Yzerfontein	33°20′S, 18°02′E	11
	SWIO	Mossel Bay	34°09′S, 22°10′E	13
	Unknown	–	–	16
Galeorhinus galeus (*N* = 24)	SEAO	Robben Island	33°48′S, 18°24′E	7
		False Bay	34°10′S, 18°36′E	7
	SWIO	Struis Bay	34°47′S, 20°03′E	3
		Mossel Bay	34°09′S, 22°10′E	2
		Port Elizabeth	34°04′S, 25°03′E	5
Triakis megalopterus (*N* = 32)	SEAO	Cape Point	34°20′S, 18°33′E	8
		Betty's Bay	34°22′S, 18°55′E	8
	SWIO	Port Elizabeth	34°04′S, 25°03′E	16

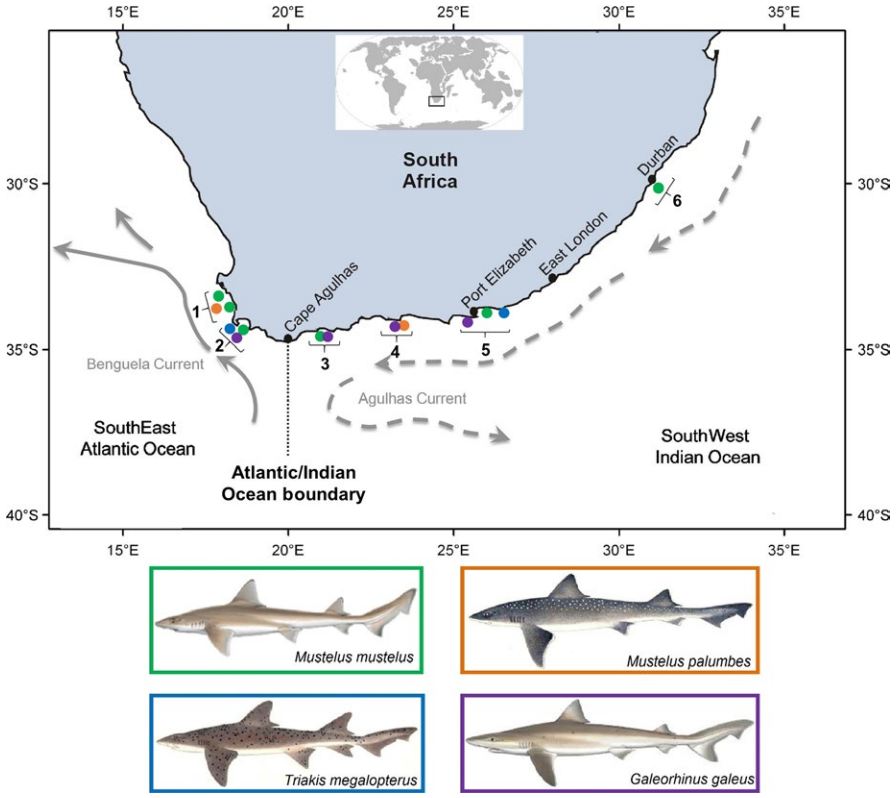

FIGURE 2 Sampling localities of four coastal shark species with the green circle representing *Mustelus mustelus*, and orange, blue, and purple circles representing *Mustelus palumbes*, *Triakis megalopterus*, and *Galeorhinus galeus*, respectively. Locations 1–2 and 3–6 represent the South African Southeast Atlantic and Southwest Indian Ocean sampled populations, respectively. The major oceanographic features are also shown

2.2 | Development of species-specific microsatellites

Total genomic DNA from one individual of common smooth-hound was isolated and sent to the Agricultural Research Council Biotechnology Platform in Pretoria, South Africa. One microgram of genomic DNA was used for 2 × 250 bp paired-end library preparation with a mean insert size of 400 bp using the standard Nextera™ library preparation kit (Illumina). The library was sequenced on two lanes of an Illumina HiSeq™ 2000 sequencer. The generated sequence reads were submitted to a quality control (QC) step to remove artificial duplicates and/or reads that contained any "Ns" using PRINSEQ 0.20.4 (Schmieder & Edwards, 2011). Reads were quality-filtered and trimmed to remove all Nextera adapters and sequences shorter than 35 bp using TRIMMOMATIC v. 0.33 (Bolger, Lohse, & Usadel, 2014) with default settings. We selected a Phred quality score of 15 and filtered for sequences that contained at least 90% of the individual bases above this quality score. To check whether primer, barcode, and adapter sequences have been properly trimmed, we visualized the sequencing quality using the software FASTQC v. 0.11.4 (Andrews, 2010). After the QC step, we built contigs from read files using ABYSS v. 1.5.2 (Simpson et al., 2009) and selected contigs larger than 250 bp for microsatellite identification in MISA v. 1.0 (Thiel, Michalek, Varshney, & Graner, 2003). Sequences with ≥5 uninterrupted motifs toward the middle were selected and blasted against the NCBI database to filter for the contigs which contained hits with microsatellites against other elasmobranch or teleost species. Sequences with hits were selected for primer design using PRIMER3 v. 0.4.0 (Untergrasser et al., 2012).

2.3 | Microsatellite validation, cross-species amplification, and species identification

Polymerase chain reaction (PCR) was carried out on a GeneAmp® PCR System 2700 in a 10 μL reaction volume that included 50 ng of template DNA, 1× PCR buffer, 200 μmol/L of each dNTP, 0.2 μmol/L of each primer, 1.5 mmol/L $MgCl_2$, and 0.1 U of GoTaq® DNA polymerase. The PCR cycling conditions were as follows: (1) one cycles of initial denaturation at 95°C for 2 min, (2) 35 cycles of denaturation at 94°C for 30 s, optimized annealing temperature (T_A) for 30 s, elongation at 72°C for 2 min, (3) a final elongation of one cycle at 60°C for 5 min and thereafter stored at 4°C. Optimum annealing temperature was determined by experimental standardization for each of the primer pairs (Table 2). Amplification products were subjected to agarose gel electrophoresis to determine their size. Levels of polymorphism were initially assessed at all the successfully amplified microsatellite loci in a panel of eight individuals of *M. mustelus*. The amplified PCR products were resolved on a vertical nondenaturing 12% polyacrylamide gel to detect size variants. We considered microsatellites to be polymorphic when two bands were distinguishable in a single individual (i.e., heterozygote), and/or we observed clear size differences between different individuals. Polymorphic microsatellite loci were selected and primers fluorescently labeled with one of the following dyes: FAM, VIC, PET, or NED followed by multiplex optimization of two mutiplex assays (MPS1 and MPS2). A panel of 48 individual *M. mustelus* representatives of the two ocean basins (SEAO and SWIO) was genotyped for marker characterization. Multiplex PCR conditions were realized using the Qiagen Multiplex PCR kit

TABLE 2 Details of 15 microsatellite loci developed for *Mustelus mustelus*

Locus name	Contig ID	Primer sequence	Motif	PCR product size	T_A (°C)	BLAST hit	PAGE results	Accession no.
Mmu1	603691 889 5405	F-CCCCATTTGCAAACAGAGTT R-ATTTCCCGCTGTTACATTGC	$(AT)_7$	210	57	*Danio rerio*	Polymorphic	KX261856
Mmu2	17731961 828 2935	F-TTGTCTGCAGGAAACACAGC R-GCATCGTGTGAAATGGGAAT	$(AC)_6$	163	56	*Cyprinus carpio*	Polymorphic	KX261857
Mmu3	19222092 770 1754	F-ATACACGGACCGACTCGAAC R-TAATGCCGAGATCAGGAACC	$(TC)_7$	240	56	*Astyanax mexicanus*	Polymorphic	KX261858
Mmu4	26403340 740 2107	F-TCCATCCAGCGTTAAAGGAC R-GCACCAGAGCTTCCCATTTA	$(TG)_7$	173	56	*Astyanax mexicanus*	Polymorphic	KX261859
Mmu5	12961358 707 1755	F-ACCACTCCCTGCAGCACTAC R-AGGAGATGCTTTGGCACTTG	$(CTC)_6$	282	57	*Callorhinchus milii*	Polymorphic	KX261860
Mmu6	14682365 1857 7998	F-CACCGGAGACCTCTAACTGG R-CGATGATGATGAAGGACGTG	$(CGC)_6$	212	57	*Chrysemys picta bellii*	Polymorphic	KX261861
Mmu7	18961995 1089 3100	F-TCCCTCATTTGCTTCAGGAG R-CGACATGAAACGCAGAAAGA	$(GCT)_5$	219	57	*Callorhinchus milii*	Polymorphic	KX261862
Mmu8	26836431 1069 3092	F-AGTAAGGCGCGCTATGATTG R-TAGAAGTCATCGCCCTCCAC	$(CAG)_5(TGT)_5$	431	56	*Callorhinchus milii*	Polymorphic	KX261863
Mmu9	7951092 402 890	F-ACGGTTCTGAGCAATCGTCT R-TGCGATATTCGTCAGGTGAA	$(GAAT)_5$	172	56	*Callorhinchus milii*	Monomorphic	KX261864
Mmu10	11748443 647 1363	F-AATCCTGAGCACCAGGACAC R-TGTGTGAATTCCCCAGATGA	$(CATA)_5$	299	56	*Squalus acanthias*	Monomorphic	KX261865
Mmu11	61216 525 1150	F-ATCTTGTTAACCGCCGACAG R-CGCCATGTTGATCGAAGTAA	$(CAA)_5$	211	56	*Callorhinchus milii*	Polymorphic	KX261866
Mmu12	1178354	F-GAGCAGCCAAGCATTAGTCC R-CGGCTTCAGAAATTGGAATC	$(GAT)_6$	208	56	*Callorhinchus milii*	Monomorphic	KX261867
Mmu13	14447036	F-TCATTCCTCACACCCACTCA R-AGATCCAGGAGCGAAGAACA	$(GCA)_5$	112	56	*Squalus acanthias*	Polymorphic	KX261868
Mmu14	12929751 492 1089	F-ACCGCTTGCTTCTGTTGAGT R-TCGCACAGACTGATTGAAGG	$(AGC)_6$	186	58	*Callorhinchus milii*	Polymorphic	KX261869
Mmu15	14824632	F-CACCTGATTGAGCAGGAGGT R-TATGGAGGTTGGGATTGCAG	$(CTC)_5$	173	58	*Squalus acanthias*	Monomorphic	KX261870

Annealing temperature in °C (T_A).

and conducted according to the manufacturer's instructions except for varying primer concentrations (Table 3) and T_A, 56°C for MPS1 and 57°C for MPS2. For subsequent analysis on an ABI 3730XL DNA Analyzer, PCR products were diluted in distilled water and fragment analysis performed together with the LIZ600 internal size standard. Individual genotypes were scored based on fragment size via GENEMAPPER v. 4.0 (Life Technologies, South Africa). To determine the utility of these markers for future regional studies of intra- and interspecific genetic diversity in houndsharks (Triakidae), we also tested the 11 microsatellite loci on the blackspotted smooth-hound, spotted gully shark, starry smooth-hound, tope shark, and whitespotted smooth-hound using the PCRs and microsatellite genotyping conditions described previously.

To evaluate the reliability of using cross-amplified microsatellites for species identification, we conducted multivariate clustering analysis using the discriminant analysis of principal components (DAPC) implemented in the R package ADEGENET (Jombart, 2008). Unlike the Bayesian clustering methods DAPC does not require specific genetic assumptions for the loci used (*e.g.*, Hardy–Weinberg and linkage equilibria). We only focused on the four coastal sharks that are commonly misidentified in South African fisheries, the common smooth-hound, spotted gully shark, tope shark, and the whitespotted smooth-hound. We performed the DAPC analysis on clusters defined by species and assessed the assignment of each individual to distinct genetic clusters using the membership coefficient, *i.e.*, the percentage of the genotype's ancestry attributed to each genetic cluster. For successful species identification, membership coefficient values had to be ≥95%.

2.4 | Microsatellite characterization

For the four study species, we tested all loci for scoring errors and allelic dropout using MICRO-CHECKER v. 2.2.3 (van Oosterhout, Hutchinson, Wills, & Shipley, 2004). The Microsatellite Excel Toolkit (MSATTOOLS v. 1.0, Park, 2001) was used to identify samples sharing identical multilocus genotypes. Duplicate genotypes with ≥95% matching alleles were excluded from further analyses. Using FREENA (Chapuis & Estoup, 2007), we estimated the frequency of null alleles following the expectation maximization (EM) method described by Dempster, Laird, and Rubin (1977). We calculated deviations from Hardy–Weinberg equilibrium (HWE) for each locus using the exact probability test based on 10,000 iterations (10,000 dememorization, 500 batches) in GENEPOP v. 4.0 (Rousset, 2008). We assessed linkage disequilibrium among loci using an exact test, also implemented in GENEPOP. False discovery rate (FDR; Benjamini & Yekutieli, 2001) control was used to adjust *p*-values for multiple comparisons (i.e., tests for departure from HWE and linkage disequilibrium) to minimize type I errors (see Narum, 2006). To test for potential signatures of selection for each locus, we used LOSITAN v. 1.44 (Antao, Lopes, Lopes, Beja-Pereira, & Luikart, 2008) with 200,000 simulations following the F_{ST} outlier method of Beaumont and Nichols (1996).

2.5 | Within-species population genetic analysis

Across sampling sites and species, we calculated the mean number of alleles per locus (N_A), allelic richness standardized for small sample size (A_R), observed heterozygosity (H_O), and heterozygosity expected under conditions of Hardy–Weinberg equilibrium (H_E) using the DIVERSITY (Keenan, McGinnity, Cross, Crozier, & Prodöhl, 2013) package for R (R Development Core Team 2015). We used MSATTOOLS to calculate the polymorphic information content (PIC) according to the equation described in Botstein, White, Skolnick, and Davis (1980). The inbreeding coefficient (F_{IS}) was calculated in ARLEQUIN v. 3.5 (Excoffier & Lischer, 2010) and tested for deviations from zero using a permutation test (1,000 permutations) with significance values adjusted using the FDR correction for multiple tests. We then used POWSIM v. 4.1 (Ryman & Palm, 2006) to assess the statistical power of the loci for F_{ST} tests (i.e., rejection of the null hypothesis H_0 of genetic homogeneity among two subpopulations when it is false) and the α level (i.e., rejection of H_0 when it is true) using a sampling scheme of two subpopulations with 20 individuals each. The analyses were conducted using 10,000 dememorizations, 100 batches, and 1,000 iterations per batch with the allele frequencies observed for the complete dataset of 11 microsatellite loci and our reported sample sizes for each species.

Pairwise F_{ST} (Weir & Cockerham, 1984) and Jost's D_{est} (Jost, 2008) were calculated using the DIVERSITY package, and the analysis of molecular variance (AMOVA) was calculated using ARLEQUIN. To account for our sampling strategy, the measures of genetic differentiation comparisons were considered significant if the lower CI was >0, and *p*-values were <.05 following FDR correction. To visualize population distinctness, we used ADEGENET to perform discriminant analysis of principal components (DAPC) on clusters defined by ocean basin. The number of clusters was assessed using the *find.clusters* function, which runs successive *K*-means clustering with increasing number of clusters (*k*). For selecting the optimal *k*, we applied the Bayesian information criterion (BIC) for assessing the best supported model, and therefore the number and nature of clusters, as recommended by Jombart, Devillard, and Balloux (2010). DAPC scatter plots were only drawn for $k > 2$. We also used a Bayesian clustering model-based method implemented in STRUCTURE 2.3 (Pritchard, Stephens, & Donnelly, 2000) to detect the most probable number of genetic clusters (*K*) present in each species. We applied an admixture model with correlated allele frequencies for 10 replicates across $K = 1$ to $K = 10$ with each run consisting of 1,000,000 Markov chain Monte Carlo (MCMC) iterations and an initial burn-in phase of 100,000 iterations assuming no prior population information. Given that only two groups of samples were compared for each species, the *ad hoc* statistic ΔK described in Evanno, Regnaut, and Goudet (2005) and commonly used to identify the likely number of genetic clusters was not considered appropriate for our study. This ΔK statistic never assigns $K = 1$ (Evanno et al., 2005). Here, the posterior probability of the data (X) for a given K, $\Pr(X|K)$, calculated by STRUCTURE was used to compute the mean likelihood $L(K)$ over 10 runs for each K to identify the likely K for which $L(K)$ was highest (Pritchard et al., 2000) as implemented in STRUCTURE

TABLE 3 Characteristics of two polymorphic microsatellite multiplex assays for *Mustelus mustelus* based on two sampling ocean basins in South Africa, Southeast Atlantic Ocean (SEAO) and Southwest Indian Ocean (SWIO)

Locus	Microsatellite repeat motif	[P]	Dye	Ocean basin	*N*	Size range (bp)	N_A	A_R	H_O	H_E	PIC	F_{IS}	P_{HW}	Fr_{NULL}
Mmu2	$(AC)_6$	0.2	FAM	SEAO	25	150–180	5	4.8	0.96	0.68	0.63	−0.391	0.000	0.000
				SWIO	23		4	3.5	0.65	0.48	0.41	−0.352	0.495	0.000
Mmu3	$(TC)_7$	0.2	NED	SEAO	25	230–250	4	3.6	0.48	0.54	0.48	0.127	0.643	0.005
				SWIO	22		6	5.2	0.41	0.65	0.59	0.393	0.054	0.121
Mmu4	$(TG)_7$	0.2	VIC	SEAO	25	158–180	8	6.3	0.88	0.67	0.61	−0.297	0.000	0.025
				SWIO	23		4	3.5	0.61	0.48	0.41	−0.260	0.368	0.000
Mmu8	$(CAG)_5(TGT)_5$	0.3	VIC	SEAO	25	417–440	8	7.1	0.72	0.78	0.75	0.096	0.710	0.016
				SWIO	21		8	6.8	0.62	0.79	0.76	0.240	0.416	0.094
Mmu11	$(CAA)_5$	0.3	PET	SEAO	24	203–209	3	3.0	0.75	0.62	0.55	−0.181	0.033	0.000
				SWIO	16		4	3.9	0.69	0.69	0.63	0.035	0.352	0.000
Mmu13	$(GCA)_5$	0.2	NED	SEAO	25	85–109	4	3.6	0.96	0.63	0.57	−0.506	0.001	0.000
				SWIO	23		5	4.5	0.78	0.57	0.53	−0.349	0.485	0.000
MPS1 (mean)	–	–	–			–	**6.5**	**5.6**	**0.714**	**0.651**	**0.588**	**−0.097**	–	**0.027**
Mmu1	$(AT)_7$	0.2	VIC	SEAO	24	200–216	5	4.9	1.00	0.69	0.64	−0.428	0.072	0.000
				SWIO	24		6	5.2	0.92	0.66	0.62	−0.369	0.443	0.000
Mmu5	$(CTC)_6$	0.3	FAM	SEAO	24	268–274	2	2.0	0.12	0.50	0.37	0.759	0.000	0.250
				SWIO	22		4	3.2	0.23	0.53	0.43	0.590	0.067	0.193
Mmu6	$(CGC)_6$	0.3	PET	SEAO	24	204–214	5	4.2	0.38	0.36	0.33	−0.035	0.000	0.000
				SWIO	21		5	4.5	0.62	0.59	0.55	−0.022	0.040	0.020
Mmu7	$(GCT)_5$	0.2	NED	SEAO	23	203–217	3	2.2	0.09	0.08	0.08	−0.011	0.997	0.000
				SWIO	24		6	5.2	0.50	0.52	0.49	0.061	0.143	0.000
Mmu14	$(AGC)_6$	0.2	FAM	SEAO	24	160–180	5	4.6	0.38	0.70	0.64	0.480	0.003	0.189
				SWIO	24		5	4.6	0.50	0.70	0.65	0.310	0.008	0.114
MPS2 (mean)	–	–	–			–	**5.8**	**4.9**	**0.471**	**0.554**	**0.496**	**0.150**	–	**0.078**
Overall (mean)	–	–	–			–	**6.2**	**5.3**	**0.604**	**0.607**	**0.546**	**0.005**	–	**0.050**

Primer concentration in the final reaction as μmol/L per primer ([P]); number of individuals (*N*); number of alleles per locus (N_A); allelic richness (A_R); observed heterozygosity (H_O); expected heterozygosity (H_E); polymorphic information content (PIC); probability of conformity to Hardy–Weinberg expectations (P_{HW}); null allele frequency (Fr_{NULL}). Mean values for each multiplex assay and overall are indicated in bold.

HARVESTER 0.6.94 (Earl & vonHoldt, 2012). CLUMPAK (Kopelman, Mayzel, Jakobsson, Rosenberg, & Mayrose, 2015) was used for the graphical representations of the STRUCTURE results. Given that we were uncertain about sampling locations of several individual *Mustelus palumbes*, we also used the program GENECLASS2 v2.0 (Piry et al., 2004), to examine genetic structure based on assignment tests for this species. Assignment probabilities of individuals were calculated using a Bayesian procedure (Rannala & Mountain, 1997) and Monte Carlo resampling using 100,000 simulated individuals and a threshold of 0.01.

Finally, we used the coalescence-based method in the program MIGRATE-N 3.6.11 (Beerli, 2006; Beerli & Palczewski, 2010) implemented on the CIPRES Portal v3.3 at the San Diego Supercomputer Center (Miller, Pfeiffer, & Schwartz, 2010) to compare alternative migration pattern across oceans. We evaluated four migration models: (1) a full model with two population sizes and two migration rates (from SEAO to SWIO and from SWIO to SEAO); (2) a model with two population sizes and one migration rate to SEAO; (3) a model with two population sizes and one migration rate to SWIO; (4) a model where SEAO and SWIO are part of the same panmictic population. The mutation-scaled effective population size $\Theta = 4N_e\mu$, where N_e is the effective population size and μ is the mutation rate per generation per locus, and mutation-scaled migration rates $M = m/\mu$, where m is the immigration rate per generation, among populations were also calculated in MIGRATE-N. A Brownian process was used to model microsatellite mutations. The Metropolis–Hastings algorithm was used to sample from the prior distributions and generate posterior distributions. Each model was run using random genealogy and values of the parameters Θ and M produced by F_{ST} calculation as a start condition. Bayesian search strategy was conducted using the following parameters: an MCMC search of 5×10^5 burn-in steps followed by 5×10^6 steps with parameters recorded every 20 steps. The prior distribution for the parameters was uniform with Θ and migration boundaries defined after explorative runs. A static heating scheme with four different temperatures (1.0, 1.5, 3.0, and 1×10^6) was employed, where acceptance–rejection swaps were proposed at every step. The model comparison was made using log-equivalent Bayes factors (LBF) that need the accurate calculation of marginal likelihoods. These likelihoods were calculated using thermodynamic integration in MIGRATE-N. Models were ordered by LBF, and the model probability (P_{Mi}) was calculated in R. Additionally, we converted estimates of gene flow (M) to the number of effective migrants (N_em) from population i to population j using the formula:

$$N_e^{(j)} m_{i\to j} = \frac{\Theta_j M_{i\to j}}{4}$$

3 | RESULTS

3.1 | Microsatellite multiplex assays, cross-species amplification, and species identification

The two sequencing runs of the Nextera™ library for *Mustelus mustelus* generated 35 GB of raw reads. After trimming the raw sequences that included removal of adapters, N-containing reads, and low-quality reads, we retained a total of 17 GB clean reads. After the *de novo* assembly of the Illumina paired-end reads, we recovered a total of 27,512,666 contigs. We identified a total of 82,879 contigs that were longer than 250 bp, of which 2,572 (3.1%) contained microsatellites. Dinucleotide repeats were the most frequent (1,629 or 86.1%), followed by trinucleotide repeats (232 or 12.3%), and tetranucleotide repeats (31 or 1.6%). We selected 15 microsatellite containing contigs for primer design with an expected PCR product size ranging between 112 and 431 bp. Of the 15 loci tested, all were successfully amplified while only 11 were polymorphic based on initial screening via polyacrylamide gels (Table 2). These loci were fluorescently labeled to construct a 5-plex and 6-plex assay that were both validated over 48 individuals from two populations of the common smooth-hound (Figures A1 and A2, Appendix).

The genetic diversity summary statistics for both multiplex assays are presented in Table 2. All markers were polymorphic and produced a total of 74 alleles (mean 6.2). There was no evidence of stutter products or significant allelic dropout based on the MICRO-CHECKER results, but null alleles were detected at two loci (Mmu5 and Mmu14) with high frequencies estimated in FREENA relative to the rest of the loci (Table 3). After correcting for multiple tests, all loci were in agreement with HWE except for Mmu5 and Mmu14 possibly due to null alleles. Linkage disequilibrium was not found between any of the loci pairs tested. The F_{ST}-outlier test showed that locus Mmu7 did not conform to selective neutrality and was under putative directional selection. The PIC ranged from 0.08 to 0.76, and the H_O and H_E ranged from 0.09 to 1 and 0.08 to 0.79, respectively. The F_{IS} value ranged from −0.506 to 0.759. Subsequent estimates of population genetic structure were therefore computed using a subset of eight microsatellites, excluding loci not conforming to Hardy–Weinberg equilibrium, neutrality, and/or exhibiting high null allele frequencies (Mmu5, Mmu7, and Mmu14). To assess the cross-species utility of the two multiplexes, we tested these assays on six other triakid species, and cross-species amplification rate of success ranged from 72% to 100% (Table 4).

Additionally, to validate the potential of these markers for within-species population genetic analysis, we inferred genetic variation in samples collected from two different ocean basins for each respective species (Table 1). In each species, all 11 microsatellites were variable with up to a mean N_A of 8, A_R up to 7.5, H_E and PIC as high as 0.842 (Tables A1, A3, and A3, Appendix). After correcting for multiple tests, all loci in each species conformed to HWE and no evidence for LD between any of the loci pairs were found. MICRO-CHECKER indicated the presence of null alleles at locus Mmu11 for the tope shark and locus Mmu4 for the spotted gully shark. Using the F_{ST}-outlier test, we only found evidence for two loci (Mmu 2 and Mmu11) putatively subjected to selection in the whitespotted smooth-hound possibly due to issues surrounding small sample sizes. Assessment of the power of the multilocus dataset to detect population structure indicated that all loci used could accurately detect differentiation as low as $F_{ST} = 0.003$, for a population sample of $n = 20$, indicating that the dataset was suitable for population structure inference.

TABLE 4 Cross-species transferability results of 11 microsatellites tested among six triakid species

Locus/species	*Galeorhinus galeus* (*N* = 8)	*Mustelus asterias* (*N* = 3)	*M. mosis* (*N* = 2)	*M. palumbes* (*N* = 8)	*M. punctulatus* (*N* = 3)	*Triakis megalopterus* (*N* = 8)
Mmu1	+ (3)	+ (1)	+ (4)	+ (3)	+ (4)	+ (2)
Mmu2	+ (3)	+ (2)	+ (1)	+ (2)	+ (1)	+ (2)
Mmu3	+ (2)	+ (3)	+ (3)	+ (2)	+ (3)	+ (2)
Mmu4	+ (2)	+ (4)	+ (2)	+ (3)	+ (4)	+ (2)
Mmu5	+ (2)	+ (2)	+ (1)	+ (2)	+ (2)	+ (2)
Mmu6	+ (2)	+ (1)	+ (1)	+ (2)	–	+ (2)
Mmu7	+ (2)	+ (1)	+ (2)	+ (3)	–	+ (2)
Mmu8	+ (4)	+ (2)	+ (1)	+ (5)	+ (2)	+ (2)
Mmu11	+ (2)	+ (1)	+ (1)	+ (2)	+ (3)	+ (2)
Mmu13	+ (3)	+ (2)	+ (2)	+ (3)	+ (6)	+ (3)
Mmu14	+ (2)	+ (2)	+ (3)	+ (2)	–	+ (2)

–, no visible band or faint bands with insufficient band intensity for scoring alleles were observed; +, solid bands with sufficient intensity for scoring alleles were detected, and in brackets the number of alleles per locus are shown.

The novel microsatellite loci demonstrated potential application in the identification of the study species. The results from the multivariate clustering analysis (DAPC) clearly depict four genetic clusters representative of each species with limited overlap (Figure 3). Here, individuals assigned to one of the four genetic clusters with a membership coefficient of >95%.

3.2 | Population genetic structure and gene flow

3.2.1 | Common smooth-hound *Mustelus mustelus*

The pairwise population differentiation indices (F_{ST} = 0.029, D_{est} = 0.021) and AMOVA (F_{ST} = 0.029, Table A4) indicated the presence of shallow population genetic structure between SEAO and SWIO (i.e., lower 95% confidence intervals >0, and *p*-values <.05 after FDR corrections). The DAPC analysis including location prior revealed two clear genetic clusters corresponding to ocean basins, whereas excluding location prior using the *find.clusters* function, the DAPC analysis identified the presence of five genetic clusters (*k* = 5) in the dataset based on the BIC score (Figure 4). The postprocessing of the STRUCTURE results using *L*(*K*) revealed one admixed cluster (*K* = 1) as the most likely number of groups present in the dataset (Figures A3a and A4a, Appendix). Coalescent analyses for migration model comparison highly supported (P_{Mi} = 1.0) Model 2 (*i.e.*, migration from SWIO to SEAO) and showed that Θ was highest in the SWIO (Θ = 5.870) and lowest in the SEAO (Θ = 0.790) (Tables A5 and A6).

3.2.2 | Whitespotted smooth-hound *Mustelus palumbes*

Pairwise differentiation test using F_{ST} indicated significant population differentiation estimates, which were congruent with the results obtained with Jost's D_{est} between all putative populations. Pairwise comparison of the unknown samples (in terms of sampling region) with the samples collected from the SEAO revealed low differentiation (F_{ST} = 0.021, D_{est} = 0.017, lower 95% CI > 0), higher levels when compared with the SWIO samples (F_{ST} = 0.086, D_{est} = 0.104, lower 95% CI > 0). Notably, population differentiation estimates were significantly large for Atlantic versus Indian Ocean comparisons (F_{ST} = 0.091, D_{est} = 0.155, lower 95% CI > 0). Global AMOVA results indicated within individual variation explains a greater amount of the total genetic variation, with less variation among populations (F_{ST} = 0.069, p < .01) (Table A4). The DAPC analysis including and excluding the location prior revealed three genetic clusters (*k* = 3) in the dataset based on the BIC score (Figure 5). Individual assignment test based on a Bayesian approach for mapping the origin of the unknown putative population assigned 60% of the individuals to the SEAO and the remainder to the SWIO, indicative of the possible existence of substructure in *M. palumbes*. Bayesian clustering analysis in STRUCTURE also supported the assignment of the unknown population to the SEAO and interoceanic population subdivision (Figures A3b and A4b, Appendix). The most likely number of groups present in the data was *K* = 3. All results were considered, we assumed the unknown putative population to have been sampled from the SEAO, and therefore, for the gene flow analysis, we grouped the unknown samples with the samples from the SEAO. The most probable MIGRATE-N coalescent model of population structure was the unidirectional model assuming asymmetric migration from SWIO to SEAO (P_{Mi} = 1.0). Estimates of Θ was highest in the SWIO (Θ = 19.660) and lowest in the SEAO (Θ = 0.540) (Tables A5 and A6).

3.2.3 | Tope shark *Galeorhinus galeus*

Population differentiation between the SEAO and SWIO was significantly greater than zero (F_{ST} = 0.034, lower 95% CI > 0), while similar to *M. mustelus*, Jost's D_{est} indicated less pronounced levels of

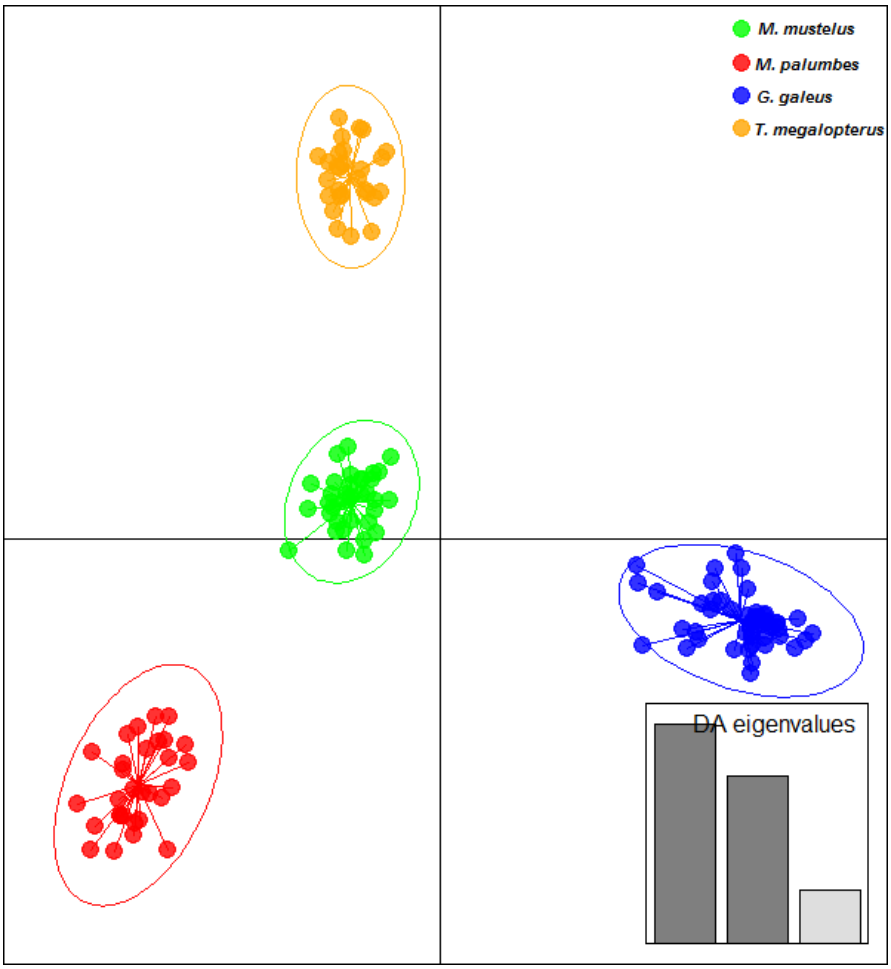

FIGURE 3 Scatterplots of DAPC analysis for a global picture of the clusters composition between species. The graph represents the individuals as dots and the groups as inertia ellipses. Eigenvalues of the analysis are displayed in inset

differentiation (D_{est} = 0.076, lower 95% CI > 0). The AMOVA results showed that there was no differentiation among populations (F_{ST} = 0.033, p = .135), but a significant amount of variance was attributed to among individuals within populations (F_{IS} = 0.093, p = .000) and within individuals (F_{IT} = 0.123, p = .000) (Table A4). The DAPC analysis including and excluding the location prior revealed two genetic clusters (k = 2) in the dataset based on the BIC score (Figure 6). Evaluation of the K values produced by STRUCTURE using the maximum value of $L(K)$ identified K = 2 as the most likely number of groups present in the data (Figures A3c and A4c, Appendix). Coalescent analyses for migration model comparison highly supported (P_{Mi} = 1.0) Model 2 (i.e., migration from SWIO to SEAO) and showed that Θ was highest in the SWIO (Θ = 98.100) and lowest in the SEAO (Θ = 0.100) (Tables A5 and A6).

3.2.4 | Spotted gully shark *Triakis megalopterus*

Based on the population differentiation estimates, there was no evidence for population subdivision between the SEAO and SWIO samples (F_{ST} = −0.012, D_{est} = −0.002, lower 95% CI < 0). The AMOVA results also showed no differentiation among populations (F_{ST} = −0.012, p = 1.000), with most of the variation explained among individuals within populations (F_{IS} = 0.134, p = .000) and within individuals (F_{IT} = 0.123, p = .000) (Table A4). The DAPC analysis showed clustering with fairly flat distributions of membership probabilities of individuals across clusters indicative of one genetic cluster in the data (Figure 7). Bayesian clustering analysis in STRUCTURE identified four admixed genetic clusters (K = 4) as the most likely number of groups present in the data (Figures A3d and A4d, Appendix). Coalescent analyses for migration model comparison highly supported (P_{Mi} = 1.0) Model 2 (i.e., migration from SWIO to SEAO) and showed that Θ was highest in the SWIO (Θ = 6.820) and lowest in the SEAO (Θ = 1.380) (Tables A5 and A6).

4 | DISCUSSION

Recent advances in next-generation sequencing technologies have considerably accelerated the mining of species-specific microsatellite loci in shark species generally devoid of molecular markers (Blower et al., 2015; Chabot & Nigenda, 2011; Pirog et al., 2015). In this study, the use of Illumina HiSeq™ 2000 for reduced genome sequencing was successful regarding speed, accuracy, and cost in generating microsatellites. It provided an efficient way to develop microsatellite markers, even though some factors such as library preparation, read length, and precision of the assembly can be improved in future studies. The

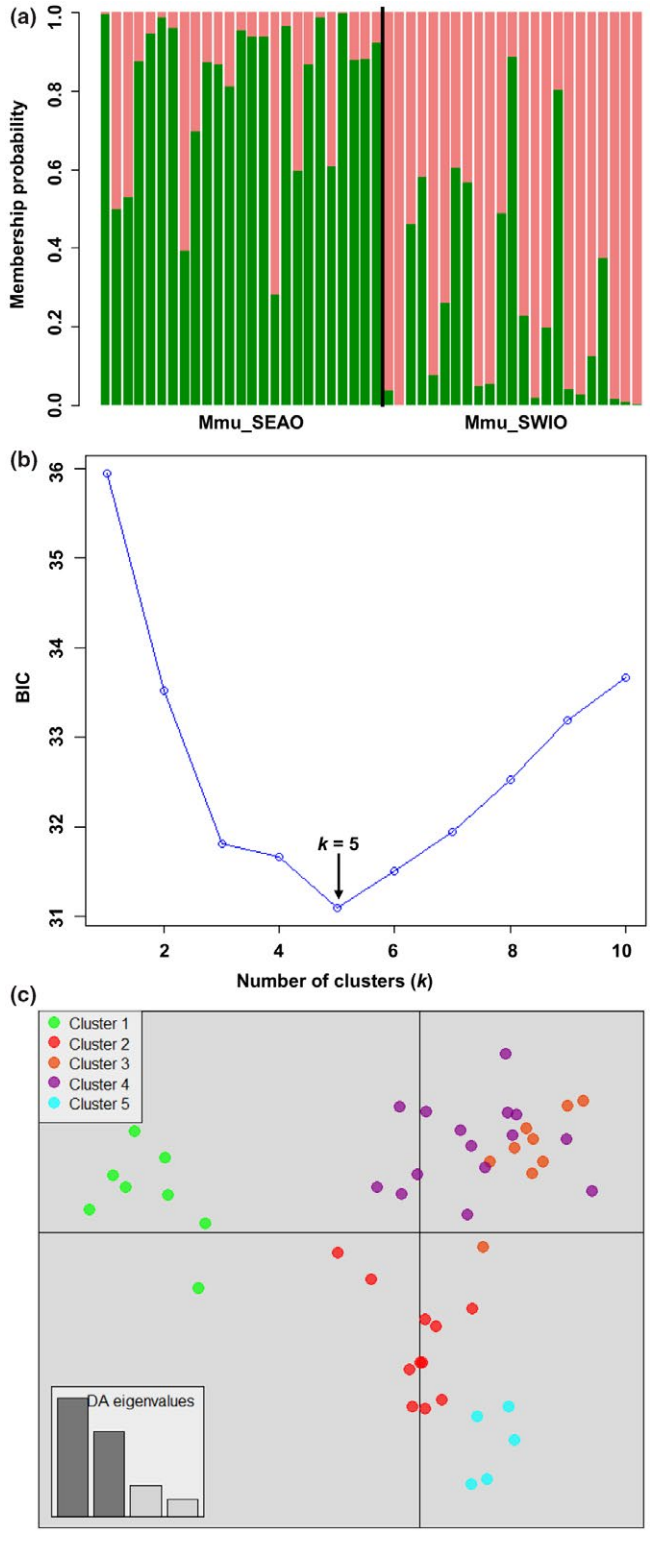

FIGURE 4 STRUCTURE-like plot, inference of the number of clusters, and scatterplots of DAPC analysis on the dataset of *Mustelus mustelus*. Mmu_SEAO and Mmu_SWIO represent the South African Southeast Atlantic and Southwest Indian Ocean sampled populations, respectively. (a) Cluster assignments by population (sampling location a priori), each individual is represented by a vertical colored line. (b) Inference of the number of clusters excluding sampling location as a priori. A *k* value of 5 (the lowest BIC value) represents the best summary of the data. (c) The graph represents the individuals as dots. Each color represents a genetic cluster (*k*)

relative richness of different types of microsatellite repeats is typical, and in sharks, dinucleotide repeats are generally overrepresented. Similar to the studies of the Australian gummy shark *Mustelus antarticus* (Boomer & Stow, 2010), the tope shark (Chabot & Nigenda, 2011), and the brown smooth-hound shark *M. henlei* (Chabot, 2012), we found that dinucleotide microsatellite repeats were the most frequent repeat type present in the common smooth-hound shark genome.

Furthermore, we successfully constructed and optimized two polymorphic multiplex assays for the common smooth-hound shark. The validation of our multiplex assays in the common smooth-hound revealed similar genetic diversity indices as found in a previous study of the same species using cross-amplified loci (Maduna et al., 2016). Given that in sharks, microsatellite flanking sequences are conserved owing to low mutation rates (Martin, Pardini, Noble, & Jones, 2002) we tested for the cross-species amplification of orthologous microsatellite loci in other Triakidae species. We observed a high cross-species amplification rate of success (>70%) across all microsatellite loci. Such findings were similar to those previously reported on sharks (Blower et al., 2015; Chabot & Nigenda, 2011; Giresi, Renshaw, Portnoy, & Gold, 2012).

There is often a negative correlation between the evolutionary distance of the focal and target species, and the transferability of loci (amplification success and polymorphism) in sharks (Maduna et al., 2014). A similar trend has also been found in several other vertebrate taxa including birds, amphibians, and fish (Carreras-Carbonell, Macpherson, & Pascual, 2007; Hendrix, Susanne Hauswaldt, Veith, & Steinfartz, 2010; Primmer, Painter, Koskinen, Palo, & Merilä, 2005). All the species that were included in this study were closely related and accordingly the high performance of cross-species amplification was expected, albeit the blackspotted smooth-hound had the lowest transferability rate possibly due to the presence of null alleles. These loci, nevertheless, could prove useful in elucidating patterns of population genetic structure and gene flow within other Triakidae species. Besides the comparison of population genetic parameters among multiple closely related species, cross-species microsatellites can also be applied for species identification based on species-specific allele sizes at multiple loci, a technique that has rarely been used for forensic studies of sharks (Giresi et al., 2015; Maduna et al., 2014; Marino et al., 2014). Indeed, our multiplex assays proved useful in discriminating between the study species, particularly for those that are morphologically very similar.

Our assessment of the distribution of genetic diversity of the four codistributed coastal sharks (the common smooth-hound, spotted gully shark, tope shark, and the whitespotted smooth-hound) based on the newly developed multiplex assays indicated that the microsatellite loci are informative for species identification as well as for population genetic analysis. Our preliminary population genetics estimates hinted at the combined effects of oceanographical barriers and life-history differences (e.g., mobility and sex-specific dispersal strategies) to be the major factors influencing the patterns of regional

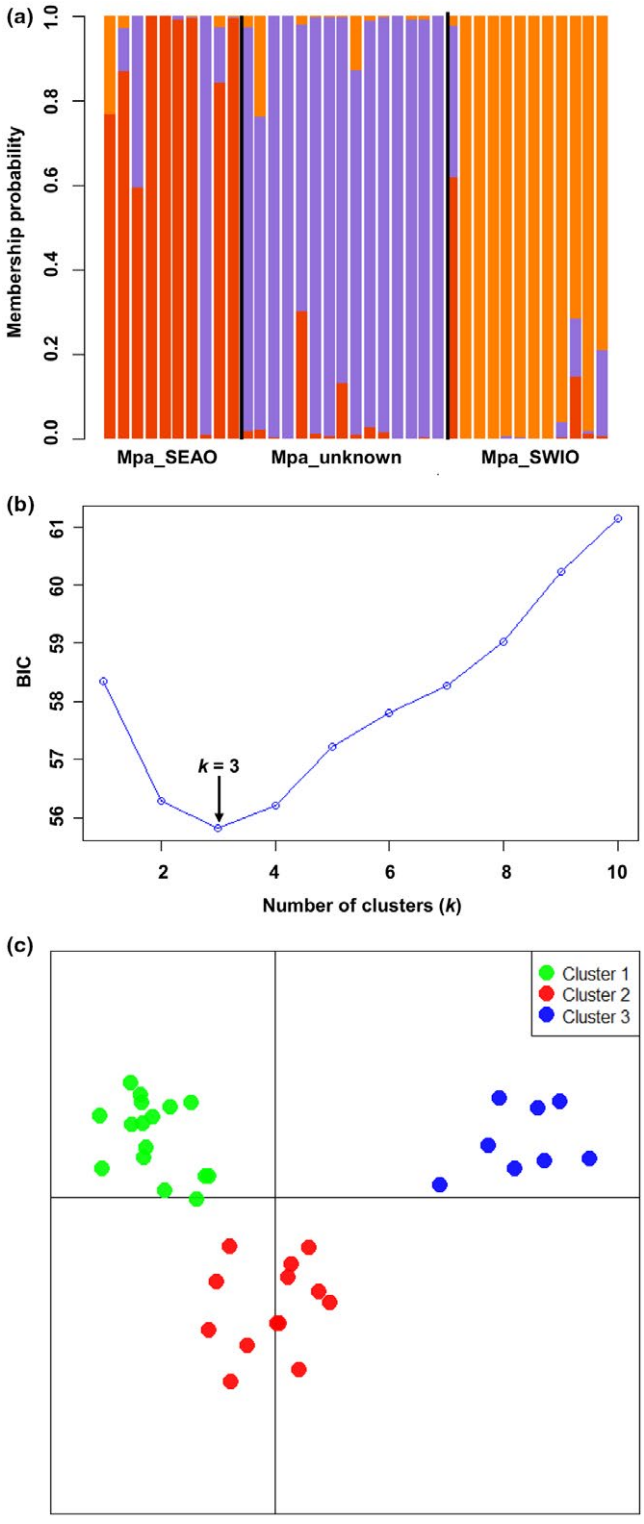

FIGURE 5 STRUCTURE-like plot, inference of the number of clusters, and scatterplots of DAPC analysis on the dataset of *Mustelus palumbes*. Mpa_SEAO and Mpa_SWIO represent the South African Southeast Atlantic and Southwest Indian Ocean sampled populations, respectively. (a) Cluster assignments by population (sampling location a priori), each individual is represented by a vertical colored line. (b) Inference of the number of clusters excluding sampling location as a priori. A *k* value of 3 (the lowest BIC value) represents the best summary of the data. (c) The graph represents the individuals as dots. Each color represents a genetic cluster (*k*)

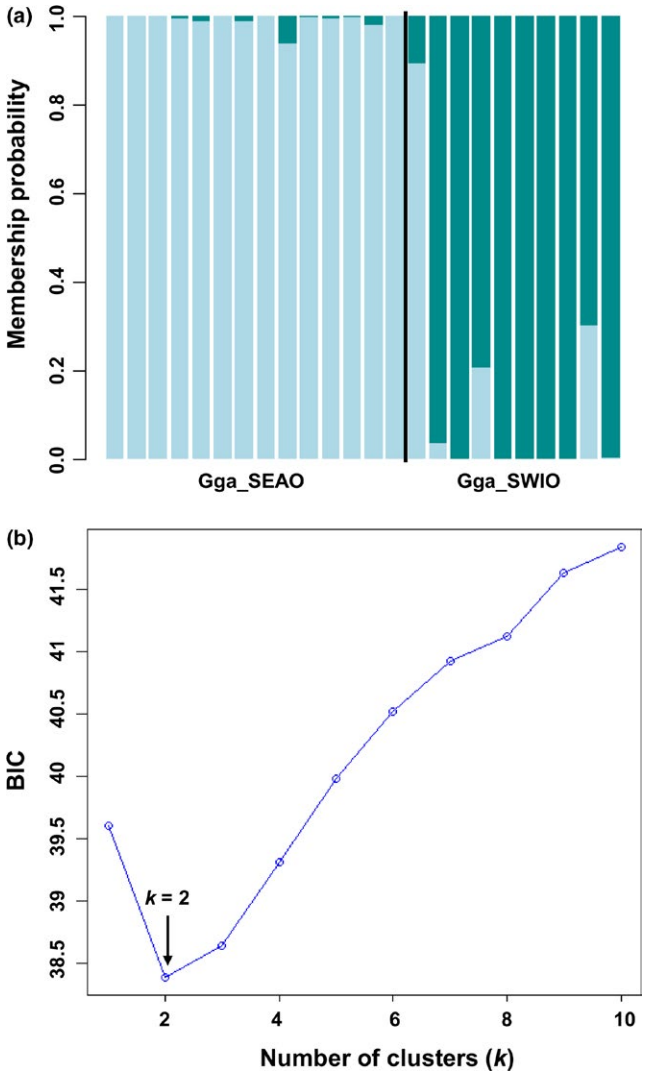

FIGURE 6 STRUCTURE-like plot, inference of the number of clusters, and scatterplots of DAPC analysis on the dataset of *Galeorhinus galeus*. Gga_SEAO and Gga_SWIO represent the South African Southeast Atlantic and Southwest Indian Ocean sampled populations, respectively. (a) Cluster assignments by population (sampling location a priori), each individual is represented by a vertical colored line. (b) Inference of the number of clusters excluding sampling location as a priori. A *k* value of 2 (the lowest BIC value) represents the best summary of the data. Each color represents a genetic cluster (*k*)

population structure in these sharks. We rejected the null hypothesis of panmixia in all the study species except for *T. megalopterus*. In line with previous studies by Bitalo et al. (2015) and Maduna et al. (2016), we detected interoceanic genetic structure in the common smooth-hound across the Atlantic/Indian Ocean boundary. Our findings also suggest the presence of fine-scale genetic structure in the whitespotted smooth-hound, indicating that the unknown sampling population was collected along a gradient of restricted gene flow. Based on the Bayesian (STRUCTURE and GENECLASS) and multivariate (DAPC) analyses, it is evident that the majority of the unknown samples came from the Atlantic Ocean. In *Mustelus* species, it seems intraspecific

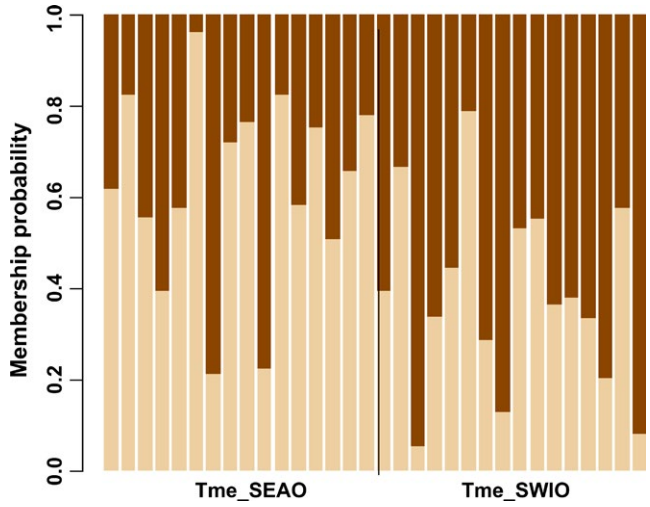

FIGURE 7 STRUCTURE-like plot of DAPC analysis on the dataset of *Triakis megalopterus*. Tme_SEAO and Tme_SWIO represent the South African Southeast Atlantic and Southwest Indian Ocean sampled populations, respectively. Each individual is represented by a vertical colored line, and each color represents a genetic cluster (*k*)

populations are typically connected via a series of stepping stone populations (Boomer, 2013; Pereyra et al., 2010). In such systems genetic structure is usually reflected by a combination of effective population size, individual movements and migrations, seascape feature, and habitat preferences, *e.g.*, the narrownose smooth-hound *M. schmitti* (Pereyra et al., 2010), the Australian gummy shark (Boomer, 2013), the rig *M. lenticulatus* (Boomer, 2013), and the brown smooth-hound shark (Chabot et al., 2015; Sandoval-Castillo & Beheregaray, 2015). Pereyra et al. (2010) and Boomer (2013) found no evidence of population genetic structure, while Chabot et al. (2015) and Sandoval-Castillo and Beheregaray (2015) provided compelling evidence for the interplay of oceanography and dispersal differential between sexes in shaping genetic structure. In agreement with Maduna et al. (2016), our study found asymmetric gene flow that predominantly occurs from the Southwest Indian Ocean to Southeast Atlantic Ocean for the common smooth-hound, and a similar trend was observed for the whitespotted smooth-hound. Granted, the reproductive and seasonal behavior of the two study smooth-hounds remain for the most part unknown (sensu Smale & Compagno, 1997; da Silva et al., 2013), particularly for the whitespotted smooth-hound, but it appears that genetic structure in these species is highly similar (at least in the samples investigated here).

Results from previous research indicated that levels of gene flow across the Atlantic/Indian Ocean boundary for the tope shark were relatively high (Bitalo et al., 2015), yet we found significant interoceanic genetic structure with two genetic clusters characterized by lower levels of admixture (SEAO and SWIO). The Bitalo et al. (2015) study, however, included only one Indian Ocean population (Struis Bay) in close proximity to the proposed boundary and noted significant population differentiation between this SWIO sampling site and a SEAO sampling site, Robben Island. In addition, Bitalo et al. (2015) did note that overall samples collected west of the Atlantic/Indian Ocean boundary exhibited a more significant level of admixture than those collected east of the boundary. We conclude that the genetic structure observed in our study is in agreement with that of the previous study given our sampling locations for the species. Similarly, for smooth-hounds, long-term gene flow estimates between ocean basins were asymmetrical and mainly occur from the Southwest Indian Ocean to Southeast Atlantic Ocean. The homogenous population structure observed here for the spotted gully shark was unexpected, given the available tagging data which indicate possible philopatric behavior for the species, although, it freely travels across the Atlantic/Indian Ocean boundary (Dunlop & Mann, 2014; Soekoe, 2016). However, it is well documented that the Atlantic/Indian Ocean boundary (Benguela Barrier) or transition zone is not fixed and extends from Cape Point (westernmost boundary) to Cape Agulhas (easternmost boundary) depending on the species in question (Teske, Von der Heyden, McQuaid, & Barker, 2011). The former may hold true for the spotted gully shark given our sampling site that we used as a representative of the Atlantic Ocean (Cape Point and Betty's Bay).

Coalescent analyses for migration model comparison highly supported the model of the southward flux of migrants (i.e., migration from SWIO to SEAO) and showed that Θ was highest in the SWIO and lowest in the SEAO populations in all study species. Our finding of similar asymmetric migration patterns in these species might suggest that such patterns arose from the action of shared physical boundaries. Also, water temperature changes have been shown to influence movement of these triakid sharks and other closely related species (Chabot & Allen, 2009; Espinoza, Farrugia, & Lowe, 2011; da Silva et al., 2013; Soekoe, 2016; West & Stevens, 2001). From the perspective of thermal physiology, albeit speculative, individuals from subtropical and/or warm-temperate bioregions can more easily colonize the cool-temperate bioregions as opposed to the reverse. Nevertheless, it is apparent that the cold Benguela Current and its interplay with the warm Agulhas Current also influence the patterns of gene flow in these coastal sharks as evident in a variety of other regional coastal fish species (Henriques, Potts, Santos, Sauer, & Shaw, 2014; Henriques, Potts, Sauer, & Shaw, 2012, 2015) as well as passively dispersing marine species (Teske, Bader, & Rao Golla, 2015). Although our population and genetic sampling are limited, the Agulhas Current presents a significant barrier to the northward migration in smaller coastal sharks. In summary, the newly developed multiplex assays will provide valuable molecular tools for species identification, assessing the distribution of genetic diversity and determining the directionality of gene flow, factors which are all vital for the conservation and management of these local exploited shark species.

ACKNOWLEDGMENTS

Anelda van der Walt is immensely acknowledged for training Simo N. Maduna and Charné Rossouw in NGS data analysis and her participation in the *in silico* screening for microsatellites. We further thank the following persons, organizations, and institutions for aiding in the acquisition of biological tissue samples (in alphabetical order): Edward Farrell, Fisheries Research Center-Salalah (Mikhail Chesalin),

KwaZulu-Natal Sharks Board, Oceans Research, South African Department of Agriculture, Forestry and Fisheries (DAFF; Melissa Goosen), South African Shark Conservancy, and White Shark Africa. This study was funded by the National Research Foundation of South Africa.

CONFLICT OF INTEREST

None declared.

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How to cite this article: Maduna SN, Rossouw C, da Silva C, Soekoe M, Bester-van der Merwe AE. Species identification and comparative population genetics of four coastal houndsharks based on novel NGS-mined microsatellites. *Ecol Evol*. 2017;7:1462–1486. https://doi.org/10.1002/ece3.2770

APPENDIX

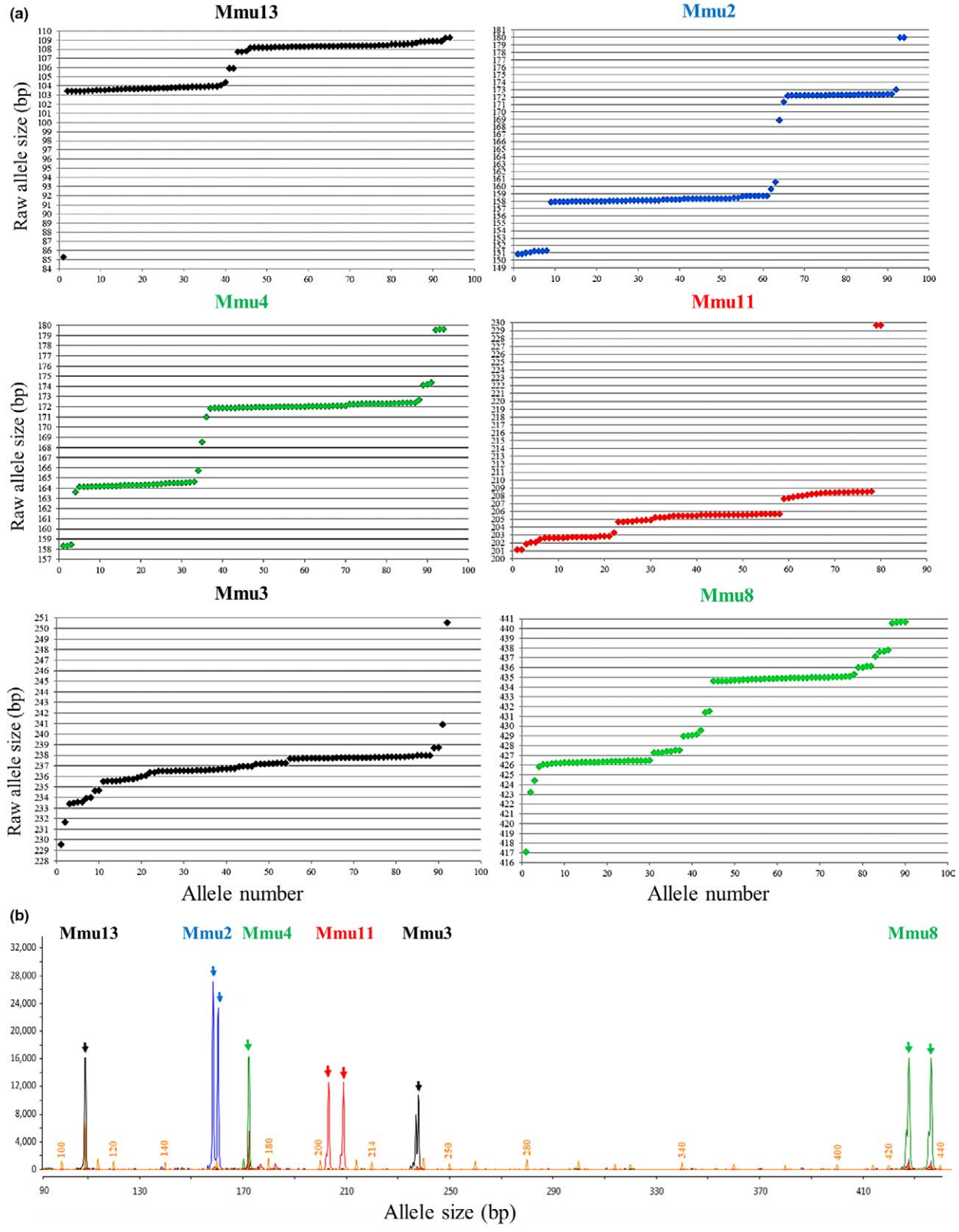

FIGURE A1 Binning and profiles for multiplex 1. (a) Allelograms based on 48 individuals from the South African South-East Atlantic and South-West Indian Ocean sampled populations, respectively. Here, the allele number corresponds to the ranking number of the allele in the list of allele raw sizes, ranked in increasing order. (b) Example of an individual electropherograms where arrows point to alleles at each locus, and small peaks with numbers (base pairs) correspond to fragments of the internal size standard LIZ600

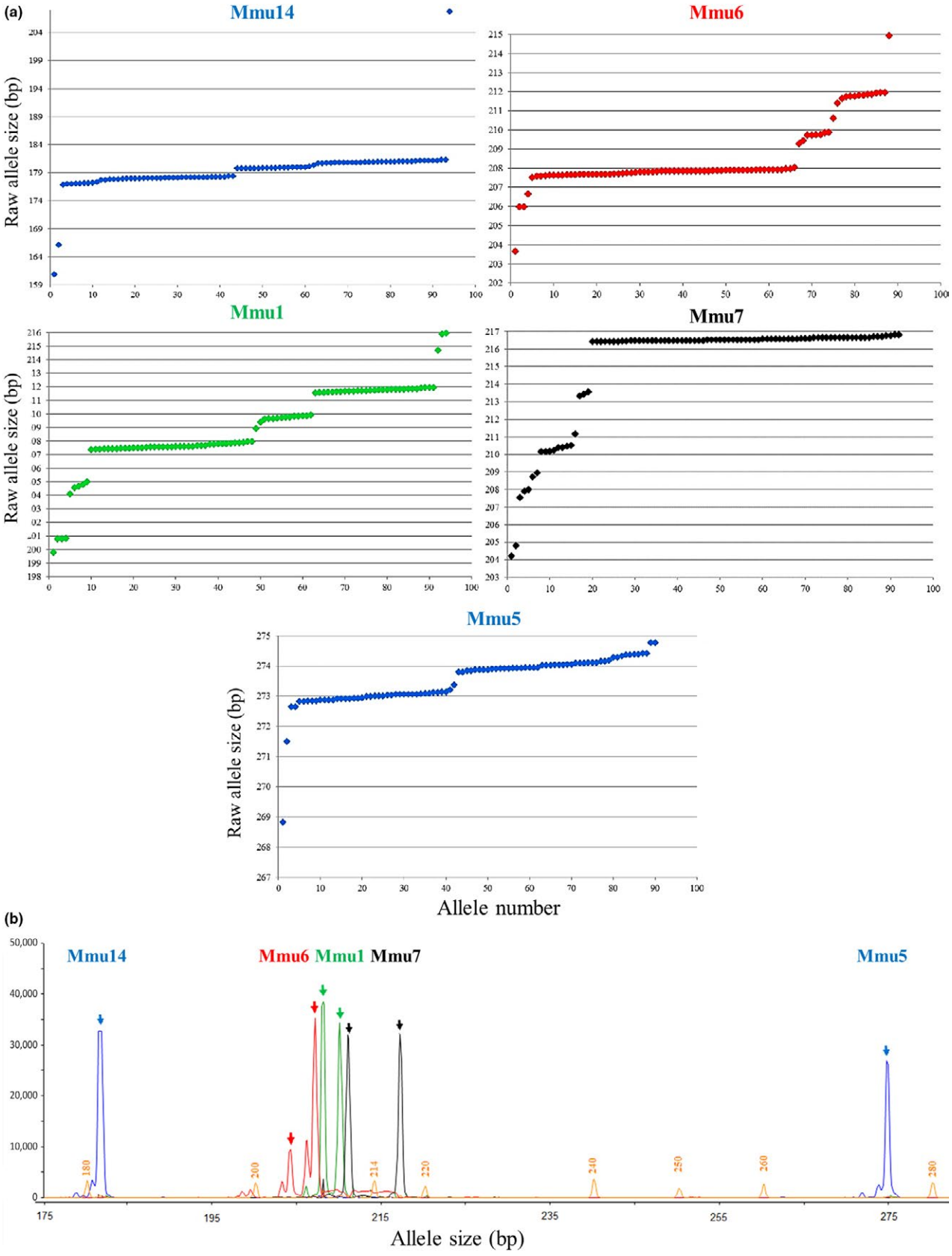

FIGURE A2 Binning and profiles for multiplex 2. (a) Allelograms based on 48 individuals from the South African South-East Atlantic and South-West Indian Ocean sampled populations, respectively. Here, the allele number corresponds to the ranking number of the allele in the list of allele raw sizes, ranked in increasing order. (b) Example of an individual electropherograms where arrows point to alleles at each locus, and small peaks with numbers (base pairs) correspond to fragments of the internal size standard LIZ600

TABLE A1 Characteristics of two polymorphic microsatellite multiplex assays for *Mustelus palumbes* based on two sampling ocean basins in South Africa, Southeast Atlantic Ocean (SEAO) and Southwest Indian Ocean (SWIO)

Locus	Microsatellite repeat motif	[P]	Dye	Ocean basin	*N*	Size range (bp)	N_A	A_R	H_O	H_E	PIC	F_{IS}	P_{HW}	Fr_{NULL}
Mmu2	$(AC)_6$	0.2	FAM	SEAO	10	139–181	4	3.3	0.40	0.36	0.33	−0.108	1.000	0.000
				Unknown	15		3	2.3	0.14	0.20	0.19	0.307	0.111	0.000
				SWIO	12		9	6.9	0.67	0.85	0.79	0.225	0.002	0.038
Mmu3	$(TC)_7$	0.2	NED	SEAO	10	228–244	5	4.5	0.60	0.70	0.62	0.150	0.025	0.067
				Unknown	15		7	6.1	0.80	0.86	0.81	0.072	0.172	0.022
				SWIO	12		5	4.4	0.60	0.76	0.67	0.217	0.238	0.074
Mmu4	$(TG)_7$	0.2	VIC	SEAO	10	157–201	2	2.0	0.30	0.27	0.22	−0.125	1.000	0.000
				Unknown	15		6	4.1	0.80	0.65	0.57	−0.235	0.000	0.018
				SWIO	12		9	6.7	0.83	0.85	0.79	0.022	0.001	0.000
Mmu8	$(CAG)_5(TGT)_5$	0.3	VIC	SEAO	10	410–438	9	7.6	0.80	0.87	0.81	0.089	0.368	0.031
				Unknown	15		2	8.0	0.85	0.90	0.85	0.057	0.525	0.000
				SWIO	12		3	7.3	0.50	0.88	0.83	0.443	0.000	0.177
Mmu11	$(CAA)_5$	0.3	PET	SEAO	10	203–212	11	1.9	0.00	0.19	0.16	1.000	0.053	0.204
				Unknown	15		1	1.0	0.00	0.00	0.00	N.A.	N.A.	0.001
				SWIO	12		7	4.4	0.10	0.74	0.65	0.871	0.000	0.353
Mmu13	$(GCA)_5$	0.2	NED	SEAO	10	85–118	9	3.0	0.43	0.67	0.55	0.379	0.329	0.135
				Unknown	15		5	4.8	0.73	0.67	0.61	−0.096	0.203	0.026
				SWIO	12		7	5.3	0.50	0.71	0.65	0.309	0.014	0.057
MPS1 (mean)	–	–	–			–	**5.8**	**4.7**	**0.503**	**0.619**	**0.561**	**0.210**	–	**0.067**
Mmu1	$(AT)_7$	0.2	VIC	SEAO	10	180–214	2	2.0	0.20	0.53	0.38	0.633	0.075	0.200
				Unknown	15		6	4.8	0.73	0.71	0.65	−0.037	0.133	0.000
				SWIO	12		6	5.4	0.50	0.82	0.75	0.400	0.005	0.168
Mmu5	$(CTC)_6$	0.3	FAM	SEAO	10	253–291	3	2.9	0.30	0.62	0.49	0.526	0.061	0.199
				Unknown	15		6	4.5	0.54	0.66	0.60	0.196	0.159	0.012
				SWIO	12		7	5.3	0.58	0.77	0.70	0.252	0.001	0.103
Mmu6	$(CGC)_6$	0.3	PET	SEAO	10	203–213	4	4.0	0.50	0.76	0.67	0.357	0.034	0.124
				Unknown	15		6	5.0	0.64	0.73	0.67	0.127	0.611	0.018
				SWIO	12		6	5.7	0.56	0.81	0.74	0.328	0.012	0.129
Mmu7	$(GCT)_5$	0.2	NED	SEAO	10	201–219	4	3.5	0.56	0.58	0.48	0.036	1.000	0.000
				Unknown	15		6	4.7	0.64	0.62	0.57	−0.045	0.555	0.000
				SWIO	12		8	6.5	0.92	0.85	0.79	−0.080	0.071	0.000

(Continues)

TABLE A1 (Continued)

Locus	Microsatellite repeat motif	[P]	Dye	Ocean basin	*N*	Size range (bp)	N_A	A_R	H_O	H_E	PIC	F_{IS}	P_{HW}	Fr_{NULL}
Mmu14	$(AGC)_6$	0.2	FAM	SEAO	10	163–209	4	3.8	0.40	0.67	0.58	0.419	0.013	0.147
				Unknown	15		6	4.7	0.67	0.74	0.67	0.097	0.183	0.028
				SWIO	12		12	9.2	0.83	0.93	0.88	0.109	0.125	0.044
MPS2 (mean)	–	–	–			–	**5.7**	**4.8**	**0.571**	**0.720**	**0.641**	**0.221**	–	**0.078**
Overall (mean)	–	–	–			–	**5.8**	**4.7**	**0.534**	**0.665**	**0.597**	**0.215**	–	**0.072**

Primer concentration in the final reaction as μmol/L per primer ([P]); number of individuals (*N*); number of alleles per locus (N_A); allelic richness (A_R); observed heterozygosity (H_O); expected heterozygosity (H_E); polymorphic information content (PIC); probability of conformity to Hardy–Weinberg expectations (P_{HW}); null allele frequency (Fr_{NULL}). Mean values for each multiplex assay and overall are indicated in bold.

TABLE A2 Characteristics of two polymorphic microsatellite multiplex assays for *Galeorhinus galeus* based on two sampling ocean basins in South Africa, Southeast Atlantic Ocean (SEAO) and Southwest Indian Ocean (SWIO)

Locus	Microsatellite repeat motif	[P]	Dye	Ocean basin	*N*	Size range (bp)	N_A	A_R	H_O	H_E	PIC	F_{IS}	P_{HW}	Fr_{NULL}
Mmu2	$(AC)_6$	0.2	FAM	SEAO	14	126–172	9	6.9	0.57	0.80	0.75	0.295	0.006	0.101
				SWIO	10		9	8.4	0.90	0.91	0.85	0.012	0.164	0.000
Mmu3	$(TC)_7$	0.2	NED	SEAO	14	230–248	7	5.8	0.79	0.79	0.72	0.000	0.104	0.000
				SWIO	10		5	5.0	1.00	0.80	0.72	−0.268	0.064	0.000
Mmu4	$(TG)_7$	0.2	VIC	SEAO	14	162–176	5	4.6	0.71	0.74	0.67	0.037	0.004	0.000
				SWIO	10		8	7.3	0.90	0.87	0.80	−0.038	0.081	0.000
Mmu8	$(CAG)_5(TGT)_5$	0.3	VIC	SEAO	11	405–436	10	8.5	0.82	0.90	0.84	0.095	0.007	0.000
				SWIO	8		9	9.0	1.00	0.93	0.85	−0.087	0.218	0.000
Mmu11	$(CAA)_5$	0.3	PET	SEAO	13	198–236	8	6.4	0.38	0.73	0.68	0.485	0.000	0.179
				SWIO	10		11	9.7	0.60	0.93	0.87	0.365	0.002	0.147
Mmu13	$(GCA)_5$	0.2	NED	SEAO	14	89–124	8	6.7	0.93	0.81	0.76	−0.154	0.394	0.000
				SWIO	10		6	5.2	0.70	0.57	0.52	−0.235	1.000	0.000
MPS1 (mean)	–	–	–			–	**7.9**	**7.0**	**0.78**	**0.81**	**0.75**	**0.042**		**0.036**
Mmu1	$(AT)_7$	0.2	VIC	SEAO	14	192–218	10	7.8	0.79	0.88	0.83	0.106	0.571	0.000
				SWIO	10		9	7.8	0.90	0.84	0.78	−0.073	0.045	0.000
Mmu5	$(CTC)_6$	0.3	FAM	SEAO	14	263–287	7	5.2	0.43	0.65	0.60	0.350	0.032	0.113
				SWIO	10		8	7.7	0.80	0.89	0.83	0.111	0.092	0.036
Mmu6	$(CGC)_6$	0.3	PET	SEAO	14	183–220	10	8.1	0.71	0.88	0.83	0.193	0.022	0.035
				SWIO	10		8	7.3	0.60	0.87	0.80	0.321	0.058	0.138
Mmu7	$(GCT)_5$	0.2	NED	SEAO	14	200–222	9	6.6	0.64	0.73	0.68	0.127	0.199	0.029
				SWIO	10		10	8.9	0.90	0.89	0.83	−0.013	0.088	0.000
Mmu14	$(AGC)_6$	0.2	FAM	SEAO	14	165–211	6	4.5	0.57	0.60	0.54	0.046	0.360	0.000
				SWIO	10		7	6.3	0.70	0.77	0.70	0.094	0.234	0.000
MPS2 (mean)	–	–	–			–	**8.4**	**7.0**	**0.70**	**0.80**	**0.74**	**0.126**		**0.035**
Overall (mean)	–	–	–			–	**8.14**	**7.00**	**0.74**	**0.81**	**0.75**	**0.080**		**0.035**

Primer concentration in the final reaction as μmol/L per primer ([P]); number of individuals (*N*); number of alleles per locus (N_A); allelic richness (A_R); observed heterozygosity (H_O); expected heterozygosity (H_E); polymorphic information content (PIC); probability of conformity to Hardy–Weinberg expectations (P_{HW}); null allele frequency (Fr_{NULL}). Mean values for each multiplex assay and overall are indicated in bold.

TABLE A3 Characteristics of two polymorphic microsatellite multiplex assays for *Triakis megalopterus* based on two sampling ocean basins in South Africa, Southeast Atlantic Ocean (SEAO) and Southwest Indian Ocean (SWIO)

Locus	Microsatellite repeat motif	[P]	Dye	Ocean basin	*N*	Size range (bp)	N_A	A_R	H_O	H_E	PIC	F_{IS}	P_{HW}	Fr_{NULL}
Mmu2	$(AC)_6$	0.2	FAM	SEAO	16	153–181	8	7.7	0.94	0.71	0.65	−0.331	0.011	0.000
				SWIO	16		6	5.8	0.75	0.63	0.55	−0.192	0.001	0.023
Mmu3	$(TC)_7$	0.2	NED	SEAO	16	230–250	4	3.8	0.19	0.18	0.17	−0.034	1.000	0.000
				SWIO	16		4	3.9	0.19	0.24	0.22	0.211	0.065	0.000
Mmu4	$(TG)_7$	0.2	VIC	SEAO	16	164–180	4	3.9	0.19	0.29	0.27	0.357	0.075	0.114
				SWIO	16		4	3.9	0.19	0.29	0.27	0.357	0.075	0.114
Mmu8	$(CAG)_5(TGT)_5$	0.3	VIC	SEAO	16	412–426	2	2.0	0.06	0.42	0.32	0.854	0.002	0.257
				SWIO	16		4	3.9	0.13	0.34	0.31	0.636	0.001	0.146
Mmu11	$(CAA)_5$	0.3	PET	SEAO	15	148–238	7	7.0	0.40	0.59	0.54	0.328	0.042	0.123
				SWIO	15		4	4.0	0.40	0.44	0.39	0.102	0.077	0.073
Mmu13	$(GCA)_5$	0.2	NED	SEAO	16	100–116	3	2.9	0.75	0.51	0.40	−0.506	0.059	0.000
				SWIO	16		4	4.0	0.88	0.63	0.53	−0.414	0.133	0.000
MPS1 (mean)	–	–	–				**4.5**	**4.4**	**0.42**	**0.44**	**0.38**	**0.114**		**0.071**
Mmu1	$(AT)_7$	0.2	VIC	SEAO	16	202–216	3	3.0	0.69	0.64	0.54	−0.082	0.619	0.000
				SWIO	16		7	6.8	0.56	0.76	0.70	0.266	0.037	0.094
Mmu5	$(CTC)_6$	0.3	FAM	SEAO	16	271–283	4	3.9	0.13	0.59	0.49	0.794	0.000	0.278
				SWIO	16		4	3.9	0.38	0.59	0.49	0.373	0.102	0.111
Mmu6	$(CGC)_6$	0.3	PET	SEAO	16	204–218	6	5.9	0.69	0.68	0.62	−0.015	0.224	0.000
				SWIO	16		6	5.9	0.50	0.76	0.70	0.350	0.009	0.120
Mmu7	$(GCT)_5$	0.2	NED	SEAO	16	208–226	6	5.7	0.25	0.29	0.28	0.149	0.305	0.000
				SWIO	16		5	4.9	0.31	0.39	0.36	0.198	0.084	0.000
Mmu14	$(AGC)_6$	0.2	FAM	SEAO	16	177–214	5	4.8	0.25	0.24	0.22	−0.053	1.000	0.000
				SWIO	16		4	3.9	0.31	0.29	0.27	−0.087	1.000	0.000
MPS2 (mean)	–	–	–				**5**	**4.87**	**0.41**	**0.52**	**0.47**	**0.189**		**0.060**
Overall (mean)	–	–	–				**4.73**	**4.62**	**0.41**	**0.48**	**0.42**	**0.148**		**0.066**

Primer concentration in the final reaction as μmol/L per primer ([P]); number of individuals (*N*); number of alleles per locus (N_A); allelic richness (A_R); observed heterozygosity (H_O); expected heterozygosity (H_E); polymorphic information content (PIC); probability of conformity to Hardy–Weinberg expectations (P_{HW}); null allele frequency (Fr_{NULL}). Mean values for each multiplex assay and overall are indicated in bold.

Species	Source of variation	Variation (%)	*F* statistic	*p* Value
Mustelus mustelus	Among populations	2.9	F_{ST} = 0.029	.006**
	Within populations	−13.7	F_{IS} = −0.147	1.000
	Within individuals	110.8	F_{IT} = 0.108	1.000
Mustelus palumbes	Among populations	6.9	F_{ST} = 0.069	.000**
	Within populations	17.5	F_{IS} = 0.188	.000**
	Within individuals	75.6	F_{IT} = 0.244	.000**
Galeorhinus galeus	Among populations	3.4	F_{ST} = 0.033	.135
	Within populations	8.9	F_{IS} = 0.093	.000**
	Within individuals	87.7	F_{IT} = 0.123	.000**
Triakis megalopterus	Among populations	−1.2	F_{ST} = −0.012	1.000
	Within populations	13.6	F_{IS} = 0.134	.000**
	Within individuals	87.6	F_{IT} = 0.123	.000**

TABLE A4 Analysis of molecular variance (AMOVA) for *Mustelus mustelus*, *Mustelus palumbes*, *Galeorhinus galeus*, and *Triakis megalopterus*; *p < .05, **p < .01.

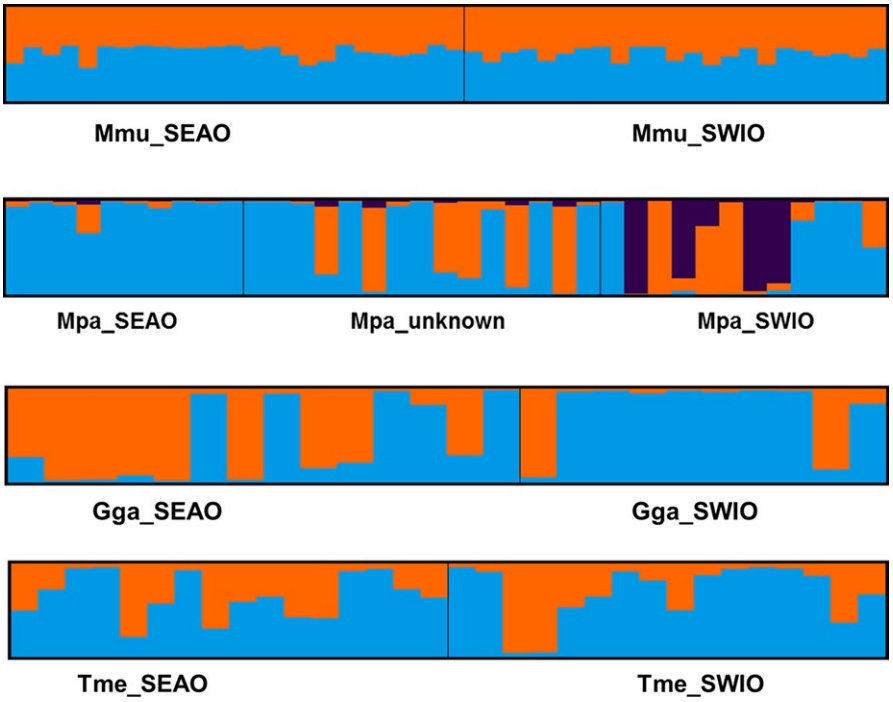

FIGURE A3 STRUCTURE results showing the most likely number of genetic clusters present in each of the four study species. SEAO and SWIO represents the South African South-East Atlantic and South-West Indian Ocean samples, respectively. Bar plots showing individual genotype membership to K clusters (each cluster is represented by a different colour, and each vertical bar represents an individual)

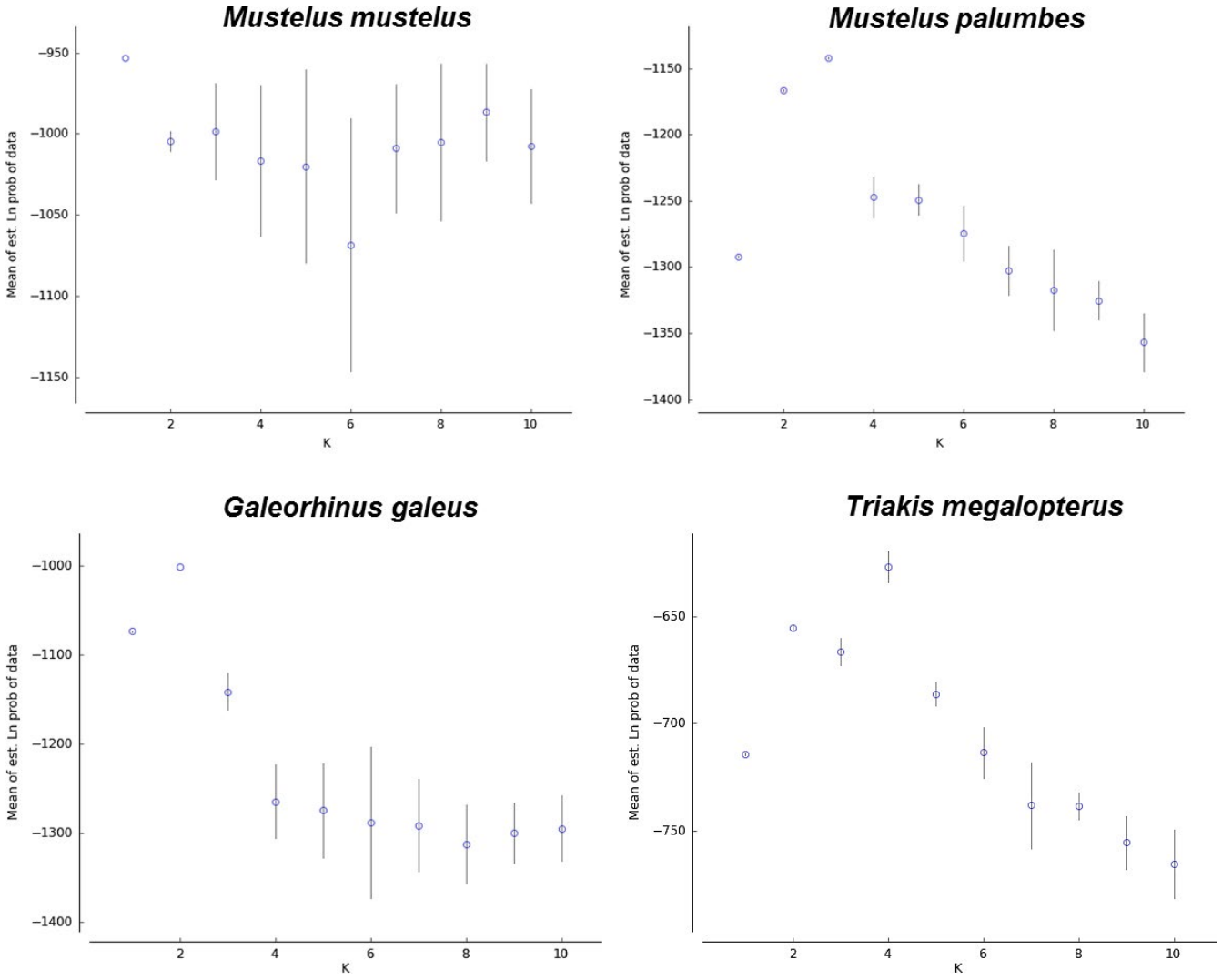

FIGURE A4 Likelihood probability profile estimated from STRUCTURE at K1-10 showing the mean and variance at each K for each study species.

TABLE A5 MIGRATE-N model selection using the approximate log marginal likelihood (lmL) method. The Bézier approximation score was used to calculate the log-equivalent Bayes factor (LBF) and select the most probable model (in bold) from among these four models. P_{Mi} is the model choice probability

Model	No. of parameters	Bézier lmL	LBF	P_{Mi}
Mustelus mustelus				
1. Full	4	−21557.47	2086.26	0.00
2. To SEAO only	**3**	**−20514.34**	**0.00**	**1.00**
3. To SWIO only	3	−20577.89	127.10	0.00
4. Panmictic	1	−29466.69	17904.70	0.00
Mustelus palumbes				
1. Full	4	−17365.13	5456.48	0.00
2. To SEAO only	**3**	**−14636.89**	**0.00**	**1.00**
3. To SWIO only	3	−14797.22	320.66	0.00
4. Panmictic	1	−21372.88	13471.98	0.00
Galeorhinus galeus				
1. Full	4	−12243.70	15635.98	0.00
2. To SEAO only	**3**	**−4425.71**	**0.00**	**1.00**
3. To SWIO only	3	−4502.19	152.96	0.00
4. Panmictic	1	−5765.09	2678.76	0.00
Triakis megalopterus				
1. Full	4	−15757.39	12746.02	0.00
2. To SEAO only	**3**	**−9384.38**	**0.00**	**1.00**
3. To SWIO only	3	−9450.22	131.68	0.00
4. Panmictic	1	−12549.03	6329.30	0.00

TABLE A6 Results from MIGRATE-N for model 2 including parameters, the mode of the posterior distribution of the migration parameter *M* and bounds of 95% confidence intervals, the Θ and N_em (product of *M* and Θ divided by 4). SEAO is the Southwast Atlantic Ocean and SWIO is the Southwest Indian Ocean basins, respectively

Species	Parameter	M mode	M 2.5%	M 97.5%	Mean
Mustelus mustelus	Θ_{SEAO}	0.79	0.40	1.32	0.86
	Θ_{SWIO}	5.87	4.94	6.88	5.91
	$M_{SWIO\rightarrow SEAO}$	37.95	29.00	49.70	38.63
	N_em	7.50			
Mustelus palumbes	Θ_{SEAO}	0.54	0.08	0.96	0.53
	Θ_{SWIO}	19.66	18.56	20.00	19.32
	$M_{SWIO\rightarrow SEAO}$	4.25	2.00	7.80	4.81
	N_em	0.57			
Galeorhinus galeus	Θ_{SEAO}	0.10	0.00	1.60	0.23
	Θ_{SWIO}	98.10	76.80	100.00	90.01
	$M_{SWIO\rightarrow SEAO}$	3.80	0.00	9.20	4.16
	N_em	0.10			
Triakis megalopterus	Θ_{SEAO}	1.38	0.28	15.36	4.77
	Θ_{SWIO}	6.82	5.64	8.04	6.85
	$M_{SWIO\rightarrow SEAO}$	89.40	52.00	146.40	96.59
	N_em	30.84			